3-9-05

AF/1637

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS & INTERFERENCES

Serial No.:	09/755,747	Group Art Unit:	1637
Filing Date:	01/05/2001	Examiner:	Fredman, J.
Applicants:	Brookes et al.	Atty. Docket:	40225.000

Title: **DETECTION OF NUCLEIC ACID POLYMORPHISM**

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APPELLANT'S APPEAL BRIEF
(37 CFR §41.37)

On Appeal From Art Unit 1637
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3-8-05	3-8-05	Maura Layton
Date of Deposit	Date of Signature	Signature

REAL PARTY IN INTEREST

The real party in interest is Anthony J. Brookes. Please note that there is an assignment, recorded at reel 12064, frame 830. assigning this application from Brookes to Hybaid Limited, a corporation organized under the laws of the England. That assignment, however, has been superceded by a subsequent written assignment (not recorded as yet) from Hybaid back to Brookes. Anthony J. Brookes is the real party in interest.

RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences.

STATUS OF CLAIMS

Claims 1-5, 7-18, 20-31, 33-44, 46-52, and 67-76 are pending. All of the claims are being appealed. All of the claims stand rejected, as follows:

Claims 1-5, 7-18, 20-31, 33-44, 46-52, and 67-76 (all of the pending claims) stand rejected under 35 USC §112, first paragraph, written description (new matter).

Claims 1-5, 8, 10-18, 21, 23-31, 34, 36-44, 47, 49-52. 67-71, 73, 74, and 76 stand rejected under 35 USC §103(a) in view of Stimpson et al. (1995) *PNAS* 92:6379-6383 and U.S. Patent 6,174,670 to Wittwer et al.:

Claims 1-5, 8, 10-18, 21, 23-31, 34, 36-44, 47, 49-52, and 67-76 stand rejected under 35 USC §103(a) in view of Stimpson et al. (1995) *PNAS* 92:6379-6383, U.S. Patent 6,174,670 to Wittwer et al., and U.S. Patent 6,048,690 to Heller et al.:

Claims 1-6, 8-19, 21-32, 34-45, 47-52, 67-71, 73, 74, and 76 stand rejected under 35 USC §103(a) in view of Stimpson et al. (1995) PNAS 92:6379-6383, U.S. Patent 6,174,670 to Wittwer et al., and U.S. Patent 5,789,167 to Konrad et al.

STATUS OF AMENDMENTS

No amendment after final rejection has been entered in the application. Appellant's response filed July 1, 2004 (in response to the Office Action dated March 1, 2004) is the last substantive amendment entered in the case and contains a full list of the claims now on appeal. See also the Claims Appendix contained hereinbelow.

SUMMARY OF CLAIMED SUBJECT MATTER

Claims 1, 14, 27, 40, 71, and 74 are independent claims. None of the claims on appeal recite "means plus function" or "step plus function" language.

All of the claims are directed to a method of detecting DNA variation by analyzing the formation or dissociation of a three-component complex. *Specification, page 2, lines 8-20 (hereinafter Spec: 2/8-20)*. The three-component complex "consists" of: (a) a single strand of sample DNA from a double stranded DNA having at least 40 base pairs, (b) an oligonucleotide or DNA probe capable of hybridizing with the single strand of DNA from (a), and an intercalating dye. *Id.* The single-stranded sample DNA is immoblized within a monolayer on a solid surface. *Spec: 2/8-20* and *Spec: 11, 9-10.* In each of the independent claims, the temperature is "steadily and progressively adjust[ed]" (Claims 1, 14, 40, 71, 74) or "steadily increas[ed]" (Claim 27) at a defined rate (0.01 to 1.0°C per second) while measuring an output signal that is indicative of the formation or the dissociation of the complex. The output signal is preferably a fluorescence signal generated by the intercalating dye. *Spec: 8/20 to 9/4.*

The utility of the invention is to detect single nucleotide differences in DNA sequences. In the preferred embodiment, a double-stranded DNA of interest (the "sample" DNA) is immobilized to a solid surface, and one of the stands is removed, thereby yielding an immobilized, single-stranded sample DNA. A probe that is specific for one variation (*i.e.*, one "allele") of the DNA of interest is then reacted with the immobilized single-stranded DNA. If the probe encounters a suitable match among the immobilized, single-stranded DNA molecules, the probe will hybridize to that immobilized DNA to yield a double-stranded DNA. Then, an intercalating dye is added which fluoresces specifically in the presence of double-

stranded DNA. That is, the dye will only intercalate with the immobilized DNA molecules that are hybridized to a complementary DNA probe (and are therefore double-stranded). The reaction temperature is then adjusted at a steady rate while continually measuring the fluorescence emitted by the dye. The temperature will eventually reach a point (the "melting temperature, T_m") where the probe DNA will dissociate from the immobilized DNA. At the T_m, there is a rapid decrease in fluorescence. Spec *8/20 to 9/22.*

In practice, an allele-specific probe is exposed to the single-stranded DNA sample. By comparing the first derivative of the signal generated by the dye (as a function of temperature) from two or more probings, it is possible to determine what alleles are present in the single-stranded DNA sample. *Spec. 9/5-10.*

Of particular note for the present appeal is that each of the independent claims requires that the immobilized, single-stranded sample DNA molecules be "within a monolayer of single DNA strands which are bound to a solid surface." The specification at page 11, lines 9 and 10 unambiguously states that "The current binding surface format used is a 96 well microtitre plate that has been coated with streptavidin (available from various manufacturers)." Further still, the specification, at page 24, line 12, explicitly states that a streptavidin-coated plate purchased from Boehringer-Mannheim was used. In the course of prosecution, Appellant submitted ample third-party evidence (both in the form of Rule 132 Declarations and product literature from third parties) showing that these streptavidin surfaces comprise monolayers. As argued in full (below), the Examiner disregarded this evidence.

Specifically addressing each of the independent claims, Claim 1 is explicitly addressed to monitoring the formation or dissociation of the complex. The complex is defined, but there is no requirement that the user form the complex. The claim presumes the existence of the complex. Claim 1 is thus a three-step method comprising: (1) steadily and progressively adjusting the temperature at a rate of between 0.01 to 1°C per second. *Spec 16/1-10.* An output signal "indicative of the interaction" (Claim 1, line 15) of the dye with duplex formed from the immobilized single-stranded DNA strand and the probe DNA is continuous measured. *Spec 16/12 to 17/17.* Lastly, the temperature at which a change in reaction output signal occurs (which is attributable to formation or dissociation of the complex) is measured.

The signal is thereby correlated with the strength with which the probe has hybridized to the single strand. *Spec 18/5 to 19/10.*

Claim 14 is similar to Claim 1, with the exception that Claim 14 positively requires that the user bring together the components needed to form the three-way complex. Thus, in Claim 14, the user must bring together a single-stranded DNA bound to a surface (*Spec: 11/8-19*), an oligonucleotide or DNA probe (*Spec: 14/12-23*), and an intercalating dye (*Spec: 14/12-23*). The components are brought together under conditions where if there is a complementary match between the sample DNA and the probe DNA, hybridization will occur. *Spec: 14/12 to 15/24.* At this point, the temperature is again steadily and progressively adjusted at a rate of between 0.01 to 1°C per second. *Spec 16/1-10.* An output signal indicative of the "extent of hybridization of [the immobilized sample DNA and the probe DNA] and the resulting complex formation with [the dye]" is continuously measured. Claim 14, clause (3) and *Spec 14/25 to 17/17.* Lastly, the temperature at which a change in reaction output signal occurs (which is attributable to formation or dissociation of the complex) is measured. *Spec 18/5 to 19/10.*

Independent Claim 27 is quite similar to Claim 14 in that Claim 27 positively requires that the user form the complex between the immobilized single-stranded DNA, the probe DNA, and the intercalating dye. See Claim 27, clause (1). The substantive difference is that Claim 27 requires "steadily increasing" the temperature (as opposed to "steadily adjusting" the temperature). See Claim 27, clause (2). In effect, Claim 27 requires that the temperature start at a low point and be steadily increased until a change in reaction output signal occurs. *Spec: 25/13-19.* Also of note is that Claim 27 requires that the change is the signal is is attributable to dissociation of the complex and is thereby correlated with the strength with which the probe hybridized to the immobilized single-stranded DNA. *Spec 18/5 to 19/10.*

Independent Claim 40 is more defined with respect to the reaction conditions as compared to Claims 14 and 27. Specifically, in the same fashion as Claim 14, Claim 40 requires that the components to form the complex be brought together. However, Claim 40 requires that the components be brought together under conditions where the probe DNA **does not** hybridize to the immobilized, single-stranded sample DNA. See Claim 40, line 13. The

temperature is then "adjusted" to cause formation of the complex. Claim 40, clause (2). The output signal is then measured and analyzed to detect the occurrence of hybridization of the probe DNA to the immobilized, single-stranded DNA. *Spec: 25/25 to 30/6.*

Independent Claim 71 is substantially identical to Claim 1, with the exception that in Claim 71, clause (a) requires that the immobilized, single-stranded DNA be "bound within a two dimensional monolayer on the surface of a solid support." In contrast, Claim 1 requires that the sample DNA be within "a monolayer of single DNA strands." Support for Claim 71 is provided in Claims 1, 7, and 9 as originally filed, *Spec: 48/1 to 49/21.*

Independent Claim 74 is substantially similar to Claims 1 and 71, with the exception that the preamble of Claim 74 explicitly recites monitoring the formation or dissociation of a "plurality" of complexes. Claim 74, line 2. Claim 74 additionally requires that the plurality of complexes form a monolayer on the surface of a solid support. *Spec: 2/8-20* and *Spec: 11, 9-10.*

Of particular note with respect to all of the independent claims is the Example starting at page 23, line 13 and extending to page 30, line 6. The Example specifically refers to Figs. 1-7, and provides a complete and exhaustively detailed working example of the present invention using three different DNA samples. The Example specifically describes amplifying the sample DNA (*Spec 23/22 to 24/7*); binding the DNA samples to the solid surface (*Spec: 24/9-13*); eluting unbound sample DNA from the solid surface (*Spec: 24/15-20*); hybridizing a first probe DNA (*Spec: 25/1-6*); removing unbound probe DNA (*Spec: 25/8-11*); detecting the signal (*Spec: 25/13-19*); re-probing the sample DNA with a different probe (*Spec: 25/21-23*); and analyzing and interpreting the data, making specific reference to the graphs presented in Figs 1-7 (*Spec: 25/25 to 30/6*).

GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The following grounds of rejection are to be reviewed on appeal:

1. The rejection of Claims 1-5, 7-18, 20-31, 33-44, 46-52, and 67-76 (all of the pending claims) under 35 USC §112, first paragraph, written description (new matter) is improper because: (a) there is no requirement that a claim recitation have *ipsis verbis* support

in the specification to comply with §112, written description; (b) the Examiner failed entirely to acknowledge or consider **on the record** the arguments and evidence presented by the Appellant's in their response dated July 1, 2004; (c) the Examiner disregarded the Strohner Declaration, which unequivocally states that solid surfaces of the type disclosed in Appellant's specification will "inevitably" yield a DNA monolayer; and (d) contrary to the Examiner's position, the Jordan et al. paper supports Appellant's position.

2. The denial of Appellant's priority claim to PCT/GB99/03329 and GB9821989.2 is improper on the same grounds as articulated in the immediately preceding paragraph.

3. The rejection of Claims 1-5, 8, 10-18, 21, 23-31, 34, 36-44, 47, 49-52. 67-71, 73, 74, and 76 under 35 USC §103(a) in view of Stimpson et al. (1995) *PNAS* 92:6379-6383 and U.S. Patent 6,174,670 to Wittwer et al. is improper because: (a) there is no motivation to combine the references; (b) even if the references are combined, they do not yield the claimed invention; and (c) the Office disregarded the contents of the Baldeschweiler Declaration (Baldeschweiler was the principal author of the Stimpson et al. paper) and the Kwok Declaration.

4. The rejection of Claims 1-5, 8, 10-18, 21, 23-31, 34, 36-44, 47, 49-52, and 67-76 under 35 USC §103(a) in view of Stimpson et al. (1995) *PNAS* 92:6379-6383, U.S. Patent 6,174,670 to Wittwer et al., and U.S. Patent 6,048,690 to Heller et al. is improper because is improper because: (a) there is no motivation to combine the references; (b) even if the references are combined, they do not yield the claimed invention; and (c) the Office disregarded the contents of the Baldeschweiler Declaration and the Kwok Declaration.

5. The rejection of Claims 1-6, 8-19, 21-32, 34-45, 47-52, 67-71, 73, 74, and 76 under 35 USC §103(a) in view of Stimpson et al. (1995) *PNAS* 92:6379-6383, U.S. Patent 6,174,670 to Wittwer et al., and U.S. Patent 5,789,167 to Konrad et al. is improper because is improper because: (a) there is no motivation to combine the references; (b) even if the references are combined, they do not yield the claimed invention; and (c) the Office disregarded the contents of the Baldeschweiler Declaration and the Kwok Declaration.

ARGUMENT

1. The Rejection of Claims 1-5, 7-18, 20-31, 33-44, 46-52, and 67-76 Under 35 USC §112, First Paragraph, Written Description (New Matter) is Improper.

(a) There is no requirement that a claim recitation have *ipsis verbis* support in the specification to comply with §112, written description.

The Office has taken the position that the term "monolayer" is regarded as new matter solely and entirely because a word search of the specification failed to locate the verbatim terms "monolayer" or "layer." See the Final Office Action dated August 9, 2004 (hereinafter " *FOA* "), at page 2, last paragraph.

The rejection is traversed on its face because the Court of Appeals for the Federal Circuit has long made it clear that there is no requirement that a claim recitation have *ipsis verbis* support in the specification to comply with §112, written description. See *Kennecott Corp. v. Kyocera International Inc.*, 5 USPQ2d 1194, 1197 (Fed. Cir. 1987). See also MPEP 2163.07(a):

> By disclosing in a patent application a device that inherently performs a function or has a property, operates according to a theory or has an advantage, a patent application necessarily discloses that function, theory or advantage, even though it says nothing explicit concerning it. The application may later be amended to recite the function, theory or advantage without introducing prohibited new matter. *In re Reynolds*, 443 F.2d 384, 170 USPQ 94 (CCPA 1971); *In re Smythe*, 480 F. 2d 1376, 178 USPQ 279 (CCPA 1973).[1]

In line with the preceding cases, the Office's insistence that the term "monolayer" is new matter for the sole reason that it does not appear verbatim in the specification is, *prima*

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[1] See also *Schering Corp. v. Amgen Inc.*, 222 F.3d 1347, 1352, 55 USPQ2d 1650, 1653 (Fed. Cir. 2000) ("The fundamental inquiry is whether the material added by amendment was inherently contained in the original application."); *TurboCare Div. of Demag Delaval Turbomachinery Corp. v. Gen. Elec. Co.*, 60 USPQ2d 1017, 1023 (Fed. Cir. 2001) ("In order for a disclosure to be inherent, 'the missing descriptive matter must necessarily be present in the application's specification such that one skilled in the art would recognize such a disclosure.') (quoting *Tronzo v. Biomet*, Inc., 156 F.3d 1154, 1159, 47 USPQ2d 1829, 1834 (Fed. Cir. 1998)).

facie, improper and should therefore be reversed. See Appellant's response of July 1, 2004, at page 14.

(b) The Examiner failed entirely to acknowledge or to consider on the record the arguments and evidence presented by the Appellant in the response dated July 1, 2004.

The Examiner states "The response [Appellant's response dated July 1, 2004] confines itself to the bare statement that 'no new matter has been added by the amendments or new claims...' but no specific support for the term monolayer is identified in the response." *FOA*, page 2, bottom. This statement is simply not true. It disregards without further comment large swaths of Appellant's July 1, 2004 response (hereinafter the "July 1 response").

The Board's attention is respectfully directed to page 14 to 17 of Appellant's July 1 response and Exhibits 1-8 submitted with the response. Appellant respectfully submits that all of these items were given short-shrift by the Examiner.

At issue is the term "monolayer," and whether the term as used in the claims presents new matter. In response to the new matter rejection over the word "monolayer," Appellant explicitly cited to page 11, lines 9 and 10 and of the specification, which states "The current binding surface format used is a 96 well microtitre plate that has been coated with streptavidin **(available from various manufacturers)**." Emphasis added. Appellant then supported the commercial availability of these streptavidin-coated surfaces by providing product literature for six (6) different commercially available streptavidin-coated surfaces. All of these commercially-available, streptavidin-coated surfaces comprise monolayers of streptavidin adhered to the surface. See Appellant's July 1 response at pages 15 and 16.

The Examiner perfunctorily dismissed the exhibits, stating "The specification did not name specific manufacturers or specific formulations, but rather generically referred to a particular plate." See *FOA*, page 3, middle. This statement is **incorrect**. The specification, at page 24, line 12, explicitly states that a streptavidin-coated plate purchased from Boehringer-Mannheim was used. See Appellant's July 1 response, page 16, second full paragraph. In a corporate merger/restructuring, Boehringer-Mannheim was renamed Roche

Molecular Biochemicals on March 5, 1998 (see Exhibit 8 to Appellant's July 1 response), which company was then subsequently re-named Roche Applied Science. The product formerly marketed under the Boehringer-Mannheim name is now sold by Roche Applied Science under the trademark StreptaWell (see Exhibit 7). The facts on this point are incontrovertible: Appellant used a plate purchased from Boehringer-Mannheim, as is clearly stated the same in the specification. (*Spec: 24/12.*) The Examiner simply ignored this fact.

Moreover, Appellant submitted product literature from **six (6) different** manufacturers, **including** the successor company to Boehringer-Mannheim, **all** describing identical streptavidin-coated plates. Appellant respectfully submits that if the plates are referred to in a "generic" fashion (to borrow the Examiner's phrase) it's only because these types of plates **are** a generic staple of commerce. They can be obtained from a slew of commercial suppliers. That's the entire point of Appellant's Exhibits 1-7. The plates, which are coated with a monolayer of streptavidin, are well-known to the person of ordinary skill in the art, and thus need not be described with exhaustive specificity in order to supply support for the term "monolayer" as used in the claims. Again, see *Kennecott Corp. v. Kyocera International Inc.*, 5 USPQ2d 1194, 1197 (Fed. Cir. 1987) and MPEP 2163.07(a).

Exhibit 1, in particular, bears close scrutiny. Exhibit 1 is an excerpt from the web site of Dynal Biotech (Oslo, Norway and Brown Deer, Wisconsin). Dynal is the maker of DYNABEAD®-brand separation matrices. Exhibit 1 describes Dynal's streptavidin-coated products. The excerpt explicitly states "Analysis and close calculations show that the bead-coating consists of a monlayer of covalently coupled streptavidin." In short, the Dynal product is **unquestionably** a surface modified to contain a **monolayer** of streptavidin. The Office never countered this evidence or presented a contrary example.

Promega Corporation (Madison, Wisconsin) also markets a 96-well microtitre plate of the type referenced at page 11 of the present specification. Not coincidentally, Promega's product is marketed under the trademark "SAM^2". See Exhibit 2 of Appellant's July 1 response. Exhibit 2 is the product insert literature for Promega's SAM^2-brand biotin capture membrane. Of particular note is that the very name of the product, SAM, is a well-known acronym for "self-assembled monolayer." (As evidence of the well-known nature of the SAM

acronym see Exhibit 3 of Appellant's July 1 response. Exhibit 3 is an excerpt from the www.acronymfinder.com website, a web-based resource that is widely cited by the trademark side of the U.S. Patent & Trademark Office.) In short, Promega's SAM^2-branded product is a surface that has been modified to contain a self-assembled monolayer of streptavidin molecules. The Office never countered this evidence or presented a contrary example.

A slew of other companies make equivalent streptavidin-coated surfaces wherein the streptavidin is in the form of a monolayer on the surface. Examples include Perkin Elmer (see Exhibit 4), Nunc (Exhibit 5), Upstate (Exhibit 6), and Roche Applied Science (Exhibit 7). All of these products are commercially available and readily located by a person of ordinary skill in the art. They are all surfaces modified to contain an immobilized streptavidin monolayer. The Office never countered this evidence.

The Examiner did perfunctorily acknowledge Appellant's exhibits in the Final Office Action, stating only that "Applicant also attempts to rely upon art, not necessarily demonstrated as prior art, which was not disclosed in the specification, and to which any reference in the specification was not limited [*sic*]." *FOA 3/7-10.* This statement does not make sense. The specification explicitly states that 96-well microtitre plates coated with streptavidin are "available from various manufacturers." *Spec: 11/9-10.* In the examples, the specification **explicitly** states that plates from Boehringer-Mannheim were used. *Spec: 24/12.* Appellant provided evidence to the Examiner clearly demonstrating that Boehringer-Mannheim was now doing business as Roche Applied Science, and that Roche Applied Science is selling these same plates under the trademark StreptaWell (see Exhibits 7 and 8 of Appellant's July 1 response). The Office simply disregarded Appellant's entire factual showing, without comment.

This rejection should thus be reversed because Appellant has provided unrebutted evidence showing that a biotin-labeled DNA, when contacted with a monolayer of immobilized streptavidin molecules, will yield a corresponding **monolayer of DNA molecules** and that such surfaces are (and were at the time of filing) readily available in commercial markets.

(c) **The Examiner disregarded the Strohner Declaration, which unequivocally states that solid surfaces of the type disclosed in Appellant's specification will "inevitably" yield a DNA monolayer.**

In its RCE filing dated January 8, 2004, Appellant included the Rule 132 Declaration of Dr. Pavel Strohner. Dr. Strohner is a co-inventor of U.S. Patent No. 6,270,983, issued August 7, 2001, and titled "Surfaces Coated with Streptavidin/Avidin." See paragraph 1 of Dr. Strohner's declaration. Of particular relevance in Dr. Strohner's declaration is paragraph 2, which states, in full:

> 2. Regardless of coating procedure details, immobilization of streptavidin onto solid-surfaces [*sic*] (such as plastic microtiter plates and membranes) will result in a reactive strepavidin monolayer. DNA molecules which are bound to this reactive streptavidin monolayer will **inevitably form a superimposed DNA monolayer**. (Emphasis added.)

The Examiner dismissed Dr. Strohner's declaration, stating:

> The claims states 'a monolayer of single DNA strands.' The claim does not recite a monolayer of streptavidin. As a note, the Declarant's patent does not refer to monolayers either. (Office Action dated March 1, 2004, page 3, bottom.)

While the Examiner's first two sentences are factually correct, **they totally ignore what Dr. Strohner actually stated in his declaration**. Dr. Strohner's above-quoted statement is imminently clear and unambiguous: regardless of the coating process, when streptavidin is coated onto a surface such as a microtiter plate, a monolayer of streptavidin results. When that streptavidin monolayer is contacted with DNA, the result is **a superimposed DNA monolayer**. Dr. Strohner was perfectly clear: the result is a **monolayer of DNA strands** as recited in the claims.

(The Examiner's parting "note" about the content of U.S. Patent 6,270,983 is, of course, wholly irrelevant. Dr. Strohner's status as an inventor of U.S. Patent 6,270,983 was included in his declaration to show the doctor's *bona fides*. In short, Dr. Strohner **is not** a simple man off the street whose sworn statement can be dismissed perfunctorily. He is an inventor in the field of streptavidin-coated surfaces.)

Because the Examiner either misunderstood Dr. Strohner's Declaration or failed to accord it the probative weight that it deserves, Appellant respectfully submits that this rejection is improper and should be reversed.

(d) Contrary to the Examiner's position, the Jordan et al. paper supports Appellant's position.

Contrary to the continued assertion offered by the Examiner, the Jordan et al. paper clearly shows that DNA contacted with a streptavidin-coated plate yields a monolayer of DNA. The Jordan et al. reference **explicitly** says so. In short, a close reading of Jordan et al. reveals that Fig. 1 of the reference depicts a sequence of steps taken over time, **each step yielding a corresponding monolayer**. In sharp contrast to the Office's interpretation, the probe DNA is **explicitly** stated in Jordan et al. as forming a "**probe DNA monolayer**." (See Jordan et al., page 4940, right-hand column, middle of first full paragraph.) Even where the binding of other molecules (in the second and third steps of Jordan et al's protocol) leads to the formation of multiple layer assemblies, the base layer of each assembly is a P DNA "**monolayer**." Each subsequent layer is a corresponding **monolayer**. In short, the various layers depicted in Fig. 1 of Jordan et al. are simply a series of **monolayers**, one on top of the other. See Appellant's July 1 response at page 18, line 1 *et seq*.

The Examiner's comments presented in the Final Office Action of August 9, 2004 are incoherent and unsupported. For example, in the sentence bridging the bottom of page 3 to the top of page 4 of the Final Office Action, the Examiner states that Appellant's citation to figure 1 of Peluso "does not support the conclusion that attachment is inherently limited to monolayers." Applicants respectfully submit that the Examiner has discounted entirely the fact that Peluso et al. clearly shows the formation of monolayers, even though though the Examiner admits on the record that this is the case. If the Office is of the position that monolayers are not the inherent result, then the Office must provide a counter-example. As noted in the paragraphs that follow, the Jordan et al. paper clearly show the formation of monolayers.

At the bottom of page 4 of the Final Office Action, the Examiner writes:

> Applicant relies upon a single short sentence to both inherently teach monolayers and to distinguish Jordan by arguing that the result of Jordan does not teach that monolayers are not inherent.

The double-negative construction of the Examiner's statement renders it difficult to decipher. The Examiner's point is apparently that even though Jordan et al. **explicitly state** that their probe DNA formed monolayers, it might not happen every time. The Examiner provides absolutely no factual support whatsoever for this contention. Fortunately, the Jordan et al. reference (as well as Dr. Strohner's declaration) are crystal clear: DNA contacted with an immobilized streptavidin monolayer **will** yield a corresponding DNA monolayer. According to Fig. 1 of Jordan et al. and the accompanying text at page 4940, right-hand column, middle of first full paragraph, the DNA forms a "**probe DNA monolayer**." According to paragraph 2 of Dr. Strohner's declaration, this result is "inevitable."

(Additionally, the Examiner's above-quoted lead-in clause, "Applicant relies on a single short sentence... " is **factually incorrect**. As noted in the earlier sections of this argument, Appellant explicitly relied upon the statement at pages 11 **and** 24 of the specification [that suitable surfaces are available commercially and that the inventor used a coated microtiter plate purchased from Boehringer-Mannheim]; Appellant relied upon the Declaration of Dr. Strohner; Appellant relied upon the explicit teaching of Jordan et al.; and Appellant relied upon the third-party evidence proving that suitable streptavidin-coated surfaces are widely available.)

The inherent result of the methods described in the specification is a **monolayer** of single stranded DNA molecules. The Jordan et al. paper explicitly says that at page 4940, right-hand column, middle of first full paragraph. Dr. Strohner explicitly declared that the result is "inevitable." The Examiner has provided no evidence to the contrary, nor has the Examiner set forth (on the record) a cogent scientific argument to refute Appellant's factual evidence.

Appellant therefore respectfully submit that the rejection of Claims 1-5, 7-18, 20-31,33-44, 46-52 and 67-76 under 35 USC §112, first paragraph is improper. Reversal of this rejection is respectfully requested.

2. The Denial of Appellant's Priority Claim to PCT/GB99/03329 and GB9821989.2 is Improper.

The denial of Appellant's priority claim to PCT/GB99/03329 and GB9821989.2 is likewise based on the use of the word "monolayer" in the claims. See *FOA*, page 5, paragraph 4. The priority applications PCT/GB99/03329 and GB9821989.2 describe the same system as disclosed in the present application. Thus, based on the same grounds articulated in the immediately preceding section of this argument, both of these priority documents provide §112(1) support for the term "monolayer." For purposes of brevity, Appellant incorporates herein by reference the arguments made above in Section (1)(a) through (1)(d) of this brief. The present claims are therefore entitled to priority to PCT/GB99/03329 and GB9821989.2 in accordance with §119 and §365. (See also MPEP §201.11.) (See also Appellant's July 1 response at page 19, bottom.)

Specifically addressing PCT/GB99/03329, the published PCT application contains the same relevant information as in the corresponding U.S. national phase application now on appeal. The Board's attention is directed to page 8, lines 26-27, and page 20, lines 25-29 of the published PCT application. These two passages also reflect that the Appellant explicitly stated in the priority document that suitable surfaces were commercially available, and that the coated microtiter plate used in the Example was purchased from Boehringer-Mannheim.

Appellant therefore submits that the Office's continued denial of the Appellant's priority claim is improper. Reversal of this rejection is respectfully requested.

3. Claims 1-5, 8, 10-18, 21, 23-31, 34, 36-44, 47, 49-52. 67-71, 73, 74, and 76 stand rejected under 35 USC §103(a) in view of Stimpson et al. (1995) *PNAS* 92:6379-6383 and U.S. Patent 6,174,670 to Wittwer et al:

Contrary to the Examiner's assertion at page 11, paragraph 9 of the Final Office Action, Appellant has not argued the references separately in trying to overcome this rejection. In fact, Appellant's response of July 1 included two separate sections given over entirely to addressing the **"combined"** teaching of the references. (Emphasis in original.) The second of these two sections was explicitly titled "The Combination of Stimpson and Wittwer does not

yield the Claimed Invention." See Appellant's July 1 response, page 23, line 1 to 25. Thus, the Examiner's assertion that Appellant argued the references separately is entirely meritless.

Appellant requests that this rejection be reversed because (a) there is no motivation to combine the references; (b) even if the references are combined, they do not yield the claimed invention; and (c) the Office disregarded the contents of the Baldeschweiler Declaration (Baldeschweiler was the principal author of the Stimpson et al. paper), the Kwok Declaration, and the Mirzabekov Declaration.

(a) There is no motivation to combine Stimpson et al. with Wittwer et al.

The overall gist of the Office's position is that it would be *prima facie* obvious to one of skill in the art to employ the SYBR Green markers of Wittwer et al. in the method of Stimpson, to thereby arrive at the presently claimed invention. In attempting to overcome this rejection, Appellant addressed the motivation to combine provided by Wittwer and the motivation to combine provided by Stimpson. A *prima facie* case of obviousness cannot be shown absent some kind of motivation provided by the references themselves. *In re Geiger,* 2 USPQ2d 1276, 1278 (Fed. Cir. 1987). If no motivation is provided by the applied prior art, it can be concluded that the Examiner impermissibly used Appellant's own disclosure to hunt through the prior art for the claimed elements and to combine them as claimed. *In re Vaeck.* 20 USPQ2d 1438 (Fed. Cir. 1991). See also Appellant's July 1 response, page 22, first paragraph.

Regarding the applied references, Appellant pointed out that Stimpson et al emphasize that fluorescence-based systems are insensitive and therefore provides an alternative optical wave guide system which improves sensitivity. Stimpson et al. specifically and purposefully set out to overcome the problems associated with fluorescence by employing an optical wave guide. There is no broader disclosure or non-preferred embodiment which might teach the skilled person to disregard the entire paper and adopt a completely different approach. This is clearly evidenced by the declaration of Dr Baldeschwieler, who was senior investigator on the Stimpson paper. (Dr. Baldeschweiler's Declaration is addressed below.) The very title of the

paper clearly sets forth the entire disclosure of the Stimpson et al. paper: "Real-time detection of DNA hybridization and melting on oligonucleotide arrays **by using optical wave guides**."

As Appellant argued at page 22, last paragraph of the July 1 response, a skilled person would receive no motivation whatsoever from Stimpson to attempt to replace the optical wave guide system, which is the central subject of the paper, with a different system. Still less would a person of ordinary skill employ a system using SYBR Green as described by Wittwer et al. In short, there is no motivation or suggestion provided by either **Wittwer et al. or Stimpson et al**. to combine these two references in the first place. See Appellant's July 1 response at page 22, last two lines.

The Examiner alleges that Wittwer et al. teach that the SYBR intercalator "is useful in the particular assay employed by Stimpson." March 1, 2004 Office Action, page 9, last paragraph. Appellant replied that this conclusion was "wholly incorrect and is unsupported by **either the Wittwer or Stimpson references**," thus clearly indicating that Appellant's were not addressing the references separately. See the July 1 response at page 20, paragraph 4. Wittwer et al. employ SYBR Green in the monitoring and quantitation of PCR and has nothing whatsoever to say or suggest regarding solid-phase hybridization, which is the subject of the present claims.

Specifically, Wittwer et al. state, at column 23, lines 9-14:

> SYBR™ Green I is a preferred double-strand-specific dye for fluorescence monitoring of PCR, primarily because of superior sensitivity, arising from greater discrimination between double-stranded and single-stranded nucleic acid. SYBR™ Green I can be used in any amplification and is inexpensive. In addition, product specificity can be obtained by analysis of melting curves, as will be described momentarily.

The teaching of "superior sensitivity" is alleged to provide motivation to use SYBR Green in the Stimpson method. However, this comment is taken wholly out of the context of the Wittwer et al. patent. SYBR Green is preferred in Wittwer et al. "for fluorescence monitoring of PCR" and Example 2 of Wittwer et al. (starting at column 22, line 28) shows that SYBR Green is more sensitive than ethidium bromide and acridine orange **for this purpose,** but not in any other context.

There simply is no teaching in Wittwer et al. that SYBR Green is a "very sensitive detection molecule" or that the alleged "superior sensitivity" of SYBR Green is sufficient to allow its use in other, unrelated applications, such as in monitoring solid-phase hybridization. Indeed, even though PCR amplification necessarily results in massive amounts of amplified product in solution, the signal produced by SYBR Green is only detectable in Figure 20 of Wittwer et al. after about 20 rounds of exponential amplification. Hence, although its sensitivity is apparently better than ethidium bromide and acridine orange in monitoring PCR reactions, **there is nothing to suggest that SYBR Green has the sensitivity required for use in solid-phase hybridization of short oligonucleotides as described in the present specification**.

There is no embodiment or suggestion of an embodiment in Wittwer et al. in which SYBR Green is used for allelic discrimination, even in solution. Columns 14 and 46, which are both cited as referring to allelic discrimination, **do not use SYBR Green**, but rather employ resonance energy transfer probes. Indeed Wittwer et al. specifically state, at column 42, lines 52-59:

> When sequence-specific detection and quantification are desired, resonance energy transfer probes can be used instead of double-strand-specific DNA dyes. The Tm of hybridization probes shifts about 4-8°C if a single base mismatch is present. If a hybridization probe is placed at a mutation site, single base mutations are detectable as a shift in the probe melting temperature.

In other words, when sequence-specific detection is required, Wittwer et al. explicitly direct that resonance energy transfer probes should be used **instead of** dyes such as SYBR Green.

The closest that Wittwer et al. come to sequence-specific detection is the use of melt curves of amplified PCR products in order to distinguish PCR products **which have completely unrelated sequences**. Intercalating agents such as SYBR Green are known to have a significant effect on the stability of duplex DNA molecules and there is no teaching that subtle differences, such as a single base-pair change in otherwise identical sequences, can be distinguished using SYBR Green (and still less single base-pair differences between very small molecules immobilized on a solid surface).

It is therefore respectfully submitted that there is no motivation provided by the Wittwer et al. patent for the skilled person to use SYBR Green in the methods of Stimpson.

(b) Even if Stimpson et al. is combined with Wittwer et al., the combination does not yield the claimed invention.

Even when Stimpson et al. and Wittwer et al. are combined, the **combined** teaching of the two references **does not** provide a skilled person a reasonable expectation of success in using SYBR Green in a solid-phase method of Stimpson.

The combination fails to provide a reasonable expectation of success because Wittwer et al. use SYBR Green to monitor the amplification in solution of nucleic acids of 110 bp or larger. Stimpson et al. concerns the solid-phase hybridization of 15-mer oligonucleotides. A skilled person is not taught or given any reasonable expectation of success by the combined teaching of Stimpson et al. and Wittwer et al. whether an intercalating agent, in particular a double-strand specific intercalating agent such as SYBR Green, would bind to a short 15-mer sequence. Moreover, the combined references provide no indication that even if binding did take place, whether the sensitivity provided would be sufficient for detection over the background signals from inter-target interactions. The combined references provide absolutely zero guidance on these matters. The person of ordinary skill in the art would thus be faced with an undue amount of experimentation, with absolutely no likelihood of success. In short, replacing a selenium label (as taught by Stimpson et al.) with an intercalating agent (as taught by Wittwer et al.) involves a considerable amount of additional experimentation, with no guidance at all from the applied references (taken alone or in combination). A skilled person therefore would have no reasonable expectation of success in the absence of such further work. See Appellant's July 1 response at page 23, line 1, *et seq.*

The Office asserts that the combination of Stimpson et al. and Wittwer et al. would overcome problems with sensitivity levels in solid-phase fluorescent hybridization systems. However, this is pure speculation on the part of the Office. The evidence of Wittwer et al. that SYBR Green is slightly better than two other dyes in monitoring PCR in solution in no way provides any reasonable expectation that SYBR Green would overcome the extreme problems of low sensitivity levels in solid-phase fluorescent hybridization systems. The Office has failed entirely to address this point.

Moreover, even if an improvement in sensitivity was observed in a completely different context (*e.g.*, solution-phase PCR reactions as described by Wittwer et al.), there is nothing to indicate that this improvement would be enough to overcome the sensitivity problems of solid-phase fluorescent hybridization systems. Note that the sensitivity problem is addressed by Stimpson by **switching away from fluorescence**, and adopting light-scattering using a selenium label. See page 6379 of Stimpson, right-hand column, first paragraph. Therefore, there is simply no technological reason a skilled artisan would combine Stimpson et al. with Wittwer et al. because Stimpson approach explicitly abandons the use of dyes as being insufficiently sensitive to acquire an unambiguous signal quickly. See also Appellant's July 1 response at page 24, first full paragraph.

Moreover, even if a skilled person were to use the SYBR Green dye in the melting curve analysis of Stimpson, the **combination** still does not yield the claimed invention. See the July 1 response at page 24, paragraph 4.

It is incontrovertible that the teaching of Wittwer et al. is restricted to molecules in solution. Likewise, it is incontrovertible that Stimpson et al. describe DNA chips which have 15-mer oligonucleotides attached thereto. In contrast, the present claims require

> a single DNA strand of a double stranded DNA **of at least 40 base pairs** containing the locus of a variation, wherein said single DNA strand is within a monolayer of single DNA strands which are bound to a solid surface....

See Claim 1, emphasis added.

The skilled person **cannot** derive this feature from the teachings of either Stimpson et al. or Wittwer et al., **taken alone or in combination**. See July 1 response, page 25, lines 1-6. The combined references are simply silent on this positively recited feature of the claimed invention. Furthermore, the present application teaches that the bound probe arrangement (rather than bound target) is problematic and does not allow allelic discrimination (see page 18, line 25, to page 19, line 27 of the present specification). These problems are resolved by the claimed "bound target" arrangement. That is, in the present invention, the sample DNA is immobilized, rather than the probe.

In conclusion, there is no reasonable expectation of success if the combination is made and even if success is achieved, the combination does not teach or suggest the invention as

positively recited by the claims as amended. Therefore, this rejection is improper and should be reversed.

(c) The Office disregarded the contents of the Baldeschweiler Declaration and the Kwok Declaration.

In support of its traversal, Appellant submitted three sworn declarations under Rule 132, none of which were accorded their proper weight.

Of particular note is Dr. Baldeschweiler's Declaration. As stated in paragraph 1 of Dr. Baldeschweiler's declaration, he was the senior investigator and principal author of the Stimpson et al. paper, the primary reference cited in this rejection. As such, Dr. Baldeschweiler's comments are extraordinarily relevant to the present rejection.

Dr. Baldeschweiler's statement regarding the lack of motivation to combine Stimpson et al. with Wittwer et al. is clear, concise, and unequivocal:

> 4. Given the well-known problems with fluorescent-based assays on a solid surface, a skilled person in the field would not have been motivated to replace the signal generation mechanism described in Stimpson et al. with any kind of fluorescent system.

To reiterate a key detail, Dr. Baldeschweiler is not "John Q. Public." He is **the principal author** of the primary document the Examiner is citing (along with Wittwer et al.) in rejecting the present claims as being obvious. Dr. Baldeschweiler is therefore clearly a person of at least ordinary skill in the art and his sworn statement is tremendously probative of the unobviousness of the present invention. Appellant's respectfully submit that the Examiner has failed to accord Dr. Baldeschweiler's declaration the respect and weight it commands.

Likewise, Appellant submitted the Declaration of Pui-Yan Kwok. Dr. Kwok directly addressed the merits of the Wittwer et al. patent. For purposes of this appeal, paragraph 5 of Dr. Kwok's declaration is highly relevant:

> 5 Wittwer et al. describe a numbr of PCR based fluorescent method which *inter alia* distinguish PCR productsusing SYBR Green I and identify polymorphisms using FRET probes. The methods involve the liquid phase hybridization of amplified DNA strands either with each other or with oligonucleotide probes. None of these methods would lead a worker in the field to the expectation that allelic discrimination could b achieved, in the absence of

amplification, using oligonucleotide hybridaztion on a solid surface using a method as presently claimed.

This statement is clear, concise, and unequivocal: the Wittwer et al. patent does not supply the motivation attributed to it by the Examiner. This statement, presented in the form of a sworn Rule 132 Declaration has not been accorded its due weight by the Office.

Appellant therefore respectfully requests that this rejection be reversed.

4. Claims 1-5, 8, 10-18, 21, 23-31, 34, 36-44, 47, 49-52, and 67-76 stand rejected under 35 USC §103(a) in view of Stimpson et al. (1995) *PNAS* 92:6379-6383, U.S. Patent 6,174,670 to Wittwer et al., and U.S. Patent 6,048,690 to Heller et al.:

For purposes of brevity, the entire previous section (3(a) through 3(c)) addressing the combination of Stimpson et al. and Wittwer et al. is incorporated herein by reference. There is no reason to combine Stimpson et al. with Wittwer et al. in the first instance (see the Baldeschweiler and Kwok Declaration), and even if the combination is made, the combination does not yield the presently claimed invention.

To recap briefly, the Wittwer et al. patent is limited entirely to detection of PCR amplification products, in solution, using a dye such as SYBR Green. Contrary to the assertion made by the Office, the Wittwer et al. patent **does not** teach or suggest using this dye to detect sequence-specific differences. Quite the contrary, Wittwer et al. explicitly teaches, at column 42, lines 50-60, that when sequence-specific detection is desired resonance energy transfer probes are to be used "instead of double-strand-specific DNA dyes." There is nothing ambiguous about Wittwer's quoted statement. Wittwer et al. explicitly teach that for this type of detection, a resonance energy transfer probe is to be used, and not a dye. The Stimpson et al. approach uses a wave-guide and a selenium light-scattering label to detect differences in DNA sequence. By Stimpson's own admission, the selenium dye was selected because "the amount of fluorescence label on the surface of a [DNA] chip is quite low." Stimpson, page 6379, right-hand column. There is simply no technological reason to combine these two references because Wittwer et al. explicitly teach that the dyes **are not** suitable for detection sequence-specific differences, and Stimpson et al. explicitly teaches that the fluorophores **are not** suitable for their purposes either. Both references explicitly teach that using a dye such as

SYBR Green for detecting differences in DNA sequence **is not** likely to be successful. Therefore, there is no motivation to combine these two references in the first place.

Further combining Wittwer et al. and Stimpson et al. with the Heller et al. document does not cure the shortcomings of the Wittwer/Stimpson combination because Heller et al. also do not address using an intercalator in the context of solid-phase DNA analysis. This shortcoming is shared **by all three documents**. The three-way **combination** of Stimpson/Wittwer/Heller **does not** address using an intercalator in the context of solid-phase DNA analysis. See Appellant's July 1 response at pages 25 and 26.

The Office cites Heller for its teaching of immobilizing oligonucleotides to arrays using the biotin/streptavidin system. Appellant has already acknowledged on the record that the biotin/streptavidin interaction has been used extensively, in a host of biological assays. See the July 1 response at page 26, second full paragraph. The point, however, is that the references to Wittwer et al. and Stimpson et al. do not teach or suggest arriving at a method wherein an intercalating dye is used. Thus, the full **combination** Stimpson et al., Wittwer et al., and Heller et al. fails to render obvious the invention as claimed. *Id.*

It is therefore respectfully submitted that the rejection of Claims 1-5, 8, 10-18, 21, 23-31, 34, 36-44, 47, 49-52, and 67-76 under 35 USC §103(a) in view of Stimpson et al., Wittwer et al., and Heller et al. is improper and should be reversed.

5. Claims 1-6, 8-19, 21-32, 34-45, 47-52, 67-71, 73, 74, and 76 stand rejected under 35 USC §103(a) in view of Stimpson et al. (1995) *PNAS* 92:6379-6383, U.S. Patent 6,174,670 to Wittwer et al., and U.S. Patent 5,789,167 to Konrad et al.

This rejection is traversed on the same grounds recited in the prior two sections, both of which are incorporated herein by reference. To recap briefly, the Wittwer et al. patent is limited entirely to detection of PCR amplification products, in solution, using a dye such as SYBR Green. Contrary to the assertion made by the Office, the Wittwer et al. patent **does not** teach or suggest using this dye to detect sequence-specific differences. Quite the contrary, Wittwer et al. explicitly teaches, at column 42, lines 50-60, that when sequence-specific detection is desired resonance energy transfer probes are to be used "instead of double-strand-

specific DNA dyes." There is nothing ambiguous about Wittwer's quoted statement. Wittwer et al. explicitly teach that for this type of detection, a resonance energy transfer probe is to be used, and not a dye. The Stimpson et al. approach uses a wave-guide and a selenium light-scattering label to detect differences in DNA sequence. By Stimpson's own admission, the selenium dye was selected because "the amount of fluorescence label on the surface of a [DNA] chip is quite low." Stimpson, page 6379, right-hand column. There is simply no technological reason to combine these two references because Wittwer et al. explicitly teach that the dyes **are not** suitable for detection sequence-specific differences, and Stimpson et al. explicitly teaches that the fluorophores **are not** suitable for their purposes either. Both references explicitly teach that using a dye such as SYBR Green for detecting differences in DNA sequence **is not** likely to be successful. Therefore, there is no motivation to combine these two references in the first place. See also the Baldeschweiler and Kwok Declarations.

Combining Stimpson et al. and Wittwer et al. with Konrad et al. does not cure the fundamental shortcomings of the combined teaching of Stimpson/Wittwer. In short, the Konrad et al. patent is cited solely for its description of typical DNA hybridization conditions. But **the full combination** of Stimpson, Wittwer, and Konrad fails entirely to teach or suggest that an intercalating dye can be used in a solid-phase format method as presently claimed. This is because Wittwer et al. explicitly teach that the dyes **are not** suitable for detection sequence-specific differences, and Stimpson et al. explicitly teach that the fluorophores **are not** suitable for their purposes either. Both of these two references explicitly teach that using a dye such as SYBR Green for detecting differences in DNA sequence **is not** likely to be successful. The Konrad et al. patent is silent on the matter. Thus, there is no motivation for combining the three references, and the **combination**, if made, does not suggest the claimed invention (as argued *supra*). See Appellant's July 1 response at page 27.

Applicants therefore submit that the rejection of Claims 1-6, 8-19, 21-32, 34-45, 47-52, 67-71, 73, 74 and 76 under 35 USC §103(a) in view of Stimpson et al., Wittwer et al., and Konrad et al. is improper and should be reversed.

CONCLUSION

In view of the law and facts stated above, and the evidence now of record, it is submitted to the Board that the Office **has not** established on the record that the specification fails to provide a complete written description of the invention as broadly as it is now claimed, nor has the Office established a prima facie case of obviousness with respect to any of Claims 1-5, 7-18, 20-31, 33-44, 46-52, and 67-76. It is therefore respectfully submitted that all of the rejections now of record are untenable and should be withdrawn.

The Board is therefore respectfully requested to reverse the Examiner's position and to allow Claims 1-5, 7-18, 20-31, 33-44, 46-52, and 67-76.

Respectfully submitted,

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CLAIMS APPENDIX

1. A method of detecting DNA variation by monitoring the formation or dissociation of a complex consisting of:

(a) a single DNA strand of a double stranded DNA of at least 40 base pairs containing the locus of a variation, wherein said single DNA strand is within a monolayer of single DNA strands which are bound to a solid surface,

(b) an oligonucleotide or DNA analogue probe specific for one allele of the variation and capable of hybridizing to the single strand (a) to form a DNA duplex,

(c) an intercalating dye specific for the DNA duplex structure of (a) plus (b) which forms a complex with the duplex and reacts uniquely when interacting within the DNA duplex,

which method comprises:

(1) steadily and progressively adjusting temperature at a rate of between 0.01 to 1°C per second,

(2) continually measuring an output signal indicative of interaction of the dye with duplex formed from the strand (a) and probe (b), and

(3) recording the temperature at which a change in reaction output signal occurs which is attributable to formation or dissociation of the complex and is thereby correlated with the strength with which the probe (b) has hybridized to the single strand (a).

2. A method according to claim 1 including

(1) forming a series of two or more complexes of the kind defined, each with a probe specific for a different allele of the variation, and

(2) observing their respective denaturing or annealing conditions (e.g. denaturing or annealing temperatures) so as to distinguish alleles of the variation plus the homozygous or heterozygous state if appropriate.

3. A method according to claim 1, in which the marker is one which fluoresces when intercalated in double stranded DNA.

4. A method according to claim 3, in which the denaturing or annealing point is determined by reference to the first derivative of the fluorescence measurement curve.

5. A method according to claim 3, in which denaturing or annealing point is determined by reference to the second derivative of the fluorescence measurement curve.

7. A method according to claim 1, in which the single strand is bound to the solid surface by a biotin/streptavidin type interaction.

8. A method according to claim 1, in which the complex is formed by adding the probe and marker to the single strand in an appropriate buffer solution.

9. A method according to claim 8, in which the buffer solution is Hepes buffer having a salt concentration less than 200 mM.

10. A method according to claim 1, using a fluorescent intercalating dye, in which the dye is SYBR Green I.

11. A method according to claim 1, in which double stranded DNA is a product of PCR amplification of a target sequence.

12. A method according to claim 11, in which the PCR product is at least 100 base pairs in length.

13. A method according to claim 11, in which the PCR product is from 40 to 100 base pairs in length.

14. A method of detecting DNA variation which comprises:

(1) bringing together:

(a) a single DNA strand of a double stranded DNA of at least 40 base pairs containing the locus of a variation, wherein said single DNA strand is within a monolayer of single DNA strands which are bound to a solid surface,

(b) an oligonucleotide or DNA analogue probe specific for one allele of the variation and capable of hybridizing to the single strand (a) to form a DNA duplex,

(c) an intercalating dye specific for the DNA duplex structure of (a) plus (b) which forms a complex with the said duplex and reacts uniquely when interacting within the duplex,

thereby forming a complex consisting of the components (a), (b) and (c), wherein the components (a), (b), and (c) are brought together under conditions in which either

(i) the component (a) hybridizes to component (b) and the complex is formed with component (c), or

(ii) the components (a) and (b) do not hybridize and the complex with component (c) is not formed,

(2) thereafter steadily and progressively adjusting the temperature at a rate of between 0.01 to 1°C per second, respectively, either

(i) to denature the formed DNA duplex and cause dissociation of the complex or

(ii) to cause formation of the DNA duplex and resulting complex,

(3) continually measuring an output signal indicative of the extent of hybridization of (a) and (b) and resulting complex formation with (c), and

(4) recording the temperature at which a change of output signal occurs which is indicative of, respectively,

(i) dissociation of the complex, or

(ii) formation of the complex.

15. A method according to claim 14 which comprises
    (1) forming a series of two or more complexes of the kind defined, each with a probe specific for a different allele of the variation, and
    (2) observing their respective denaturing or annealing conditions (e.g. denaturing or annealing temperatures) so as to distinguish alleles of the variation plus the homozygous or heterozygous state if appropriate.

16. A method according to claim 14, in which the marker is one which fluoresces when intercalated in double stranded DNA.

17. A method according to claim 16, in which the denaturing or annealing point is determined by reference to the first derivative of the fluorescence measurement curve.

18. A method according to claim 16, in which denaturing or annealing point is determined by reference to the second derivative of the fluorescence measurement curve.

20. A method according to claim 14, in which the single strand is bound to the solid surface by a biotin/streptavidin type interaction.

21. A method according to claim 14, in which the complex is formed by adding the probe and marker to the single strand in an appropriate buffer solution.

22. A method according to claim 21, in which the buffer solution is Hepes buffer having a salt concentration less than 200 mM.

23. A method according to claim 14, using a fluorescent intercalating dye, in which the dye is SYBR Green I.

24. A method according to claim 14, in which the double stranded DNA is a product of PCR amplification of a target sequence.

25. A method according to claim 24, in which the PCR product is at least 100 base pairs in length.

26. A method according to claim 24, in which the PCR product is from 40 to 100 base pairs in length.

27. A method of detecting DNA variation which comprises:
    (1) forming a complex consisting of:
        (a) a single DNA strand of a double stranded DNA of at least 40 base pairs containing the locus of a variation, wherein said single DNA strand is within a monolayer of single DNA strands which are bound to a solid surface,
        (b) an oligonucleotide or DNA analogue probe specific for one allele of the variation hybridized to the single strand (a) to form a duplex, and
        (c) an intercalating dye specific for the DNA duplex structure of (a) plus (b) and which reacts uniquely when interacting within the DNA duplex, and
    (2) continually measuring an output signal of the extent of the resulting reaction of the marker and the duplex while steadily increasing the temperature at a rate of between 0.01 to 1°C per second,
    (3) recording the temperature at which a change in reaction output signal occurs which is attributable to dissociation of the complex and is thereby correlated with the strength with which the probe (b) has hybridized to the single strand (a).

28. A method according to claim 27, which comprises
    (1) forming a series of two or more complexes of the kind defined, each with a probe specific for a different allele of the variation, and
    (2) observing their respective denaturing or annealing conditions (e.g. denaturing or annealing temperatures) so as to distinguish alleles of the variation plus the homozygous or heterozygous state if appropriate.

29. A method according to claim 27, in which the marker is one which fluoresces when intercalated in double stranded DNA.

30. A method according to claim 29, in which the denaturing or annealing point is determined by reference to the first derivative of the fluorescence measurement curve.

31. A method according to claim 29, in which denaturing or annealing point is determined by reference to the second derivative of the fluorescence measurement curve.

33. A method according to claim 27, in which the single strand is bound to the solid surface by a biotin/streptavidin type interaction.

34. A method according to claim 27, in which the complex is formed by adding the probe and marker to the single strand in an appropriate buffer solution.

35. A method according to claim 34, in which the buffer solution is Hepes buffer having a salt concentration less than 200 mM.

36. A method according to claim 27, using a fluorescent intercalating dye, in which the dye is SYBR Green I.

37. A method according to claim 27, in which the double stranded DNA is a product of PCR amplification of a target sequence.

38. A method according to claim 37, in which the PCR product is at least 100 base pairs in length.

39. A method according to claim 37, in which the PCR product is from 40 to 100 base pairs in length.

40. A method of detecting DNA variation which comprises:
(1) bringing together:
(a) a single DNA strand of a double stranded DNA of at least 40 base pairs containing the locus of a variation, wherein said single DNA strand is within a monolayer of single DNA strands which are bound to a solid surface,
(b) an oligonucleotide or DNA analogue probe specific for one allele of the variation and capable of hybridizing to the single strand (a) to form a DNA duplex,
(c) an intercalating dye specific for the DNA duplex structure of (a) plus (b) and which reacts uniquely when interacting within the duplex,
the components (a), (b) and (c) being brought together prior to formation of the defined complex and under conditions in which (a) and (b) do not hybridize;
(2) steadily adjusting the temperature at a rate of between 0.01 to 1°C per second to cause formation of the duplex and resulting complex consisting of components (a), (b), and (c), and
(3) measuring an output signal indicative of the occurrence of hybridization of (a) and (b).

41. A method according to claim 40, which comprises
(1) forming a series of two or more complexes of the kind defined, each with a probe specific for a different allele of the variation, and
(2) observing their respective denaturing or annealing conditions (e.g. denaturing or annealing temperatures) so as to distinguish alleles of the variation plus the homozygous or heterozygous state if appropriate.

42. A method according to claim 40, in which the marker is one which fluoresces when intercalated in double stranded DNA.

43. A method according to claim 42, in which the denaturing or annealing point is determined by reference to the first derivative of the fluorescence measurement curve.

44. A method according to claim 42, in which denaturing or annealing point is determined by reference to the second derivative of the fluorescence measurement curve.

46. A method according to claim 40, in which the single strand is bound to the solid surface by a biotin/streptavidin type interaction.

47. A method according to claim 40, in which the complex is formed by adding the probe and marker to the single strand in an appropriate buffer solution.

48. A method according to claim 47, in which the buffer solution is Hepes buffer having a salt concentration less than 200 mM.

49. A method according to claim 40, using a fluorescent intercalating dye, in which the dye is SYBR Green I.

50. A method according to claim 40, in which the double stranded DNA is a product of PCR amplification of a target sequence.

51. A method according to claim 50, in which the PCR product is at least 100 base pairs in length.

52. A method according to claim 50, in which the PCR product is from 40 to 100 base pairs in length.

67. A method according to claim 4 comprising determining the presence, in the negative of said derivative of the fluorescent measurement curve, one or both of: a first peak associated with the denaturing of a match probe target duplex and a second peak associated with the presence of a mis-match probe target duplex.

68. A method according to claim 17 comprising determining the presence, in the negative of said derivative of the fluorescent measurement curve, one or both of: a first peak associated with the denaturing of a match probe target duplex and a second peak associated with the presence of a mis-match probe target duplex.

69. A method according to claim 30 comprising determining the presence, in the negative of said derivative of the fluorescent measurement curve, one or both of: a first peak associated with the denaturing of a match probe target duplex and a second peak associated with the presence of a mis-match probe target duplex.

70. A method according to claim 43 comprising determining the presence, in the negative of said derivative of the fluorescent measurement curve, one or both of: a first peak associated with the denaturing of a match probe target duplex and a second peak associated with the presence of a mis-match probe target duplex.

71. A method of detecting DNA variation by monitoring the formation or dissociation of a complex consisting of:

(a) a single DNA strand of a double stranded DNA of at least 40 base pairs containing the locus of a variation, bound within a two dimensional monolayer on the surface of a solid support,

(b) an oligonucleotide or DNA analogue probe specific for one allele of the variation and capable of hybridizing to the single strand (a) to form a DNA duplex, and;

(c) an intercalating dye specific for the DNA duplex structure of (a) plus (b) which forms a complex with the said duplex and reacts uniquely when interacting within the DNA duplex,

which method comprises:

(i) steadily and progressively adjusting the temperature at a rate of between 0.01 to 1°C per second,

(ii) continually measuring an output signal indicative of interaction of the dye with duplex formed from the strand (a) and probe (b), and

(iii) recording the temperature at which a change in reaction output signal occurs which is attributable to formation or dissociation of the complex and is thereby correlated with the strength with which the probe (b) has hybridized to the single strand (a).

72. The method of claim 71 wherein the single DNA strand is bound to the surface of the support by a biotin/streptavidin type interaction.

73. The method of claim 71 wherein the complex is formed by adding the probe and the marker to the single strand in a buffer having a salt concentration less than 200 mM.

74. A method of detecting DNA variation by monitoring the formation or dissociation of a plurality of complexes, each said complex consisting of:

(a) a single DNA strand of a double stranded DNA of at least 40 base pairs containing the locus of a variation,

(b) an oligonucleotide or DNA analogue probe specific for one allele of the variation and capable of hybridizing to the single strand (a) to form a DNA duplex, and;

(c) an intercalating dye specific for the DNA duplex structure of (a) plus (b) which forms a complex with the said duplex and reacts uniquely when interacting within the DNA duplex,

wherein each said complex is bound to a surface of a solid support and said plurality of complexes form a monolayer on said surface,

which method comprises:

(i) steadily and progressively adjusting the temperature at a rate of between 0.01 to 1°C per second,

(ii) continually measuring an output signal indicative of interaction of the dye with duplex formed from the strand (a) and probe (b), and

(iii) recording the temperature at which a change in reaction output signal occurs which is attributable to formation or dissociation of the complex and is thereby correlated with the strength with which the probe (b) has hybridized to the single strand (a).

75. The method of claim 74 wherein the single DNA strand is bound to the surface of the support by a biotin/streptavidin type interaction.

76. The method of claim 74 wherein the complex is formed by adding the probe and the marker to the single strand in a buffer having a salt concentration less than 200 mM.

* * *

EVIDENCE APPENDIX

The following documents are submitted herewith:

1. The Rule 132 Declaration of Pui-Yan Kwok, submitted with Appellant's first Request for Continued Examination (RCE) dated February 10, 2003. Receipt of Appellant's first RCE filing is acknowledged on the cover sheet of the Office Action dated April 8, 2003. While the Examiner **never** directly addressed the Kwok Declaration on its merits, in the Office Action dated April 8, 2003, at page 9, lines 11-12, the Examiner stated "The declaration is addressed by the understanding of the ordinary artisan, as discussed above." This comment was made with apparent reference to both the Kwok and the Mirzabekov Declarations (see next entry).

2. The Rule 132 Declaration of Pavel Strohner, submitted with Appellant's second Request for Examination dated January 8, 2004. Receipt of Appellant's second RCE filing is acknowledged on the cover sheet of the Office Action dated March 1, 2004. The Strohner Declaration is directly addressed by the Examiner at page 3, third full paragraphof the Office Action dated March 1, 2004.

3. The Rule 132 Declaration of John D. Baldeschweiler, submitted with Appellant's second Request for Examination dated January 8, 2004. Receipt of Appellant's second RCE filing is acknowledged on the cover sheet of the Office Action dated March 1, 2004. The Baldeschweiler Declaration is directly addressed by the Examiner at page 12, second full paragraph of the Office Action dated March 1, 2004.

4. Exhibits 1 through 8, submitted with Appellant's response dated July 1, 2004. Receipt of Appellant's response is acknowledged on the cover sheet of the Final Office Action dated August 9, 2004. The Examiner did not directly address this evidence in the Final Office Action.

5. Jordan et al. (12/15/1997) "Surface Plasmon Resonance Imaging Measurements of DNA Hybridization Adsorption and Streptavidin/DNA Multilayer Formation at Chemically Modified Gold Surfaces," *Anal. Chem.* 69(24):4939-4947. Jordan et al. was cited by the Examiner in the Office Action dated March 1, 2004, at the bottom of page 3 to the top of page 4. See also line U of the PTO-form 892 that accompanied the March 1, 2004 Office Action.

6. Stimpson et al. (July 1995) "Real-time detection of DNA hybridization and melting on oligonucleotide arrays by using optical wave guides," *Proc. Natl. Acad. Sci USA* 92:6379-6383. Cited by the Examiner in the Office Action dated March 1, 2004, page 6, paragraph 8.

7. Wittwer et al., U.S. Patent No. 6,147,670, issued January 16, 2001, on an application filed June 4, 1997. Cited by the Examiner in the Office Action dated March 1, 2004, page 6, paragraph 8.

8. Heller et al., U.S. Patent No. 6,048,690, issued April 11, 2000, on an application filed May 14, 1997. Cited by the Examiner in the Office Action dated March 1, 2004, page 10, paragraph 9.

9. Konrad et al., U.S. Patent No. 5,789,167, issued August 4, 1998, on an application filed February 14, 1996 (§371 date). Cited by the Examiner in the Office Action dated March 1, 2004, page 11, paragraph 10.

10. PCT Published Application WO 00/20633, the PCT application from which the present application matured. This was forwarded to the Office by WIPO at the time Appellant satisfied the requirements of 35 USC §371.

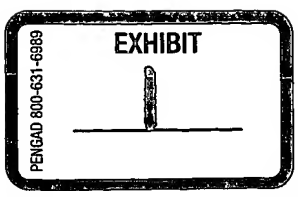
EXHIBIT
PENGAD 800-631-6989

Docket Number: 40225.000

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Brookes, et al.
Serial No.	:	09/755,747
Examiner	:	Jeffrey Fredman
Art Unit	:	1637
Filed	:	5 January 2001
For	:	DETECTION OF NUCLEIC ACID POLYMORPHISM

DECLARATION OF PUI-YAN KWOK

I, Pui-Yan Kwok, do hereby declare and state as follows.

1. I currently hold the position of Henry Bachrach Distinguished Professor at the University of California in San Francisco. I was assistant and associate professor of medicine at Washington University in St. Louis from 1993 to 2002. I have been an active researcher in the field of nucleic acid analysis and a part of the Human Genome Project since 1990. I have invented 4 DNA detection methods (3 patented) and was the editor of a recent book on the identification and detection of DNA polymorphisms. As a communicating editor of the journal Human Mutation and a member of the editorial board of several scientific journals, I am knowledgeable regarding the field of nucleic acid polymorphism detection. A copy of my curriculum vitae is attached as Exhibit 1.

2. I make this Declaration specifically to address the teachings of Drobyshev et al (Gene 188 (1997) 45 52) and Wittwer et al (US6,174,670). It is my opinion that, as of the priority date of this application, neither Drobyshev et al nor Wittwer et al, either individually or combination with each other or any other teachings then available in the art, would have allowed the ordinarily skilled artisan to perform a DASH method as described

in the specification and pending claims of the above-identified patent application or provided the artisan with a reasonable expectation of successfully discriminating polymorphic alleles by dynamic monitoring of hybridization in solid phase.

3. Prior to the development of the DASH method described in the specification, methods of hybridization involving a nucleic acid molecule bound to a solid surface (i.e. 'solid phase' hybridization) lacked the sensitivity required for the dynamic discrimination of different alleles of a nucleic acid sequence. Dynamic discrimination requires high sensitivity in order that the extent of hybridization can be followed continuously as the temperature is changed. The long exposure times required in previous methods in order to detect surface-bound signal did not allow continuous monitoring and only allowed the static determination of hybridization at a particular temperature.

4. Drobyshev et al describes hybridization of target sequence to oligonucleotides immobilized within a polyacrylamide gel. Oligonucleotides within a gel are disposed in a three-dimensional and random arrangement and an ordinarily skilled artisan would not consider that these gel-immobilized oligonucleotides are 'bound to a solid surface'. This is supported by the finding in Drobyshev et al that the hybridization of the gel-immobilized oligonucleotides resembles liquid phase rather than solid phase hybridization.

5. Wittwer et al describes a number of PCR based fluorescent methods which *inter alia* distinguish PCR products using SYBR Green I and identify polymorphisms using FRET probes. The methods involve the liquid phase hybridization of amplified DNA strands either with each other or with oligonucleotide probes. None of these methods would lead a worker in the field to the expectation that allelic discrimination could be achieved, in the absence of amplification, using oligonucleotide hybridization on a solid surface using a method as presently claimed.

6. A person working in the field before the priority date would learn nothing from the disclosures of either or both of Wittwer et al and Drobyshev et al which would enable them to perform a DASH method as described in the present claims, nor would the combined teaching of these documents impart any expectation that probe hybridization could be

monitored in real time in the solid phase with sufficient sensitivity to discriminate between alleles of a polymorphism.

7. I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: Dec 6, 2002

Pui-Yan Kwok, M.D., Ph.D.

CURRICULUM VITAE - Pui-Yan Kwok, M.D., Ph.D.

334-60-1731

December 2002

Personal Information:

Date of Birth:	January 6, 1956
Place of Birth:	Hong Kong
Citizenship:	U.S.A.
Marital Status:	Married to Abby A. Li, Ph.D.
Children:	Benjamin (7/88), Thomas (11/90), Adam (4/97)

Address and Telephone Numbers:

Office:	University of California, San Francisco 505 Parnassus Avenue Long 1332A, Box 0130 San Francisco, CA 94143-0130 415-514-3802, 415-476-2283 (FAX)
Home:	193 Saint Germain Ave San Francisco, CA 94114 415-664-1879, 415-271-7832 (Mobile)
Email:	kwok@cvrimail.ucsf.edu

Present Position:	Professor of Dermatology Henry Bachrach Distinguished Professor and Investigator, Cardiovascular Research Institute

Education and Experience:

1979	B.A.(Honors), University of Chicago, Chemistry
1981	M.S., University of Chicago, Human Biology Thesis: Integration of traditional chinese medicine and western medicine in contemporary China. Mentor: Ralph Nicholas, Ph.D.
1985	Ph.D., University of Chicago, Organic Chemistry Dissertation: Total synthesis and enzymatic studies of 10,10-difluoroarachidonic acid. Mentor: Josef Fried, Ph.D.
1987	M.D., Pritzker School of Medicine, University of Chicago
1987-1988	Intern, Department of Internal Medicine, Rush-Presbyterian-St. Luke's Medical Center, Chicago, Illinois
1988-1990	Resident, Division of Dermatology, Washington University School of Medicine, St. Louis, Missouri
1990-1991	Chief Resident, Division of Dermatology, Washington University School of Medicine, St. Louis, Missouri

1990-1992	Postdoctoral Fellow, Division of Dermatology and Department of Genetics, Washington University School of Medicine, St. Louis, Missouri. Mentor: Maynard V. Olson, Ph.D.
1992-1993	Visiting Scientist, Department of Molecular Biotechnology, University of Washington School of Medicine, Seattle, Washington. Mentor: Maynard V. Olson, Ph.D.
1993-1999	Assistant Professor of Dermatology and Genetics Washington University School of Medicine, St. Louis, MO
1999-2002	Associate Professor of Dermatology and Genetics Washington University School of Medicine, St. Louis, MO
4/2002 -	Professor of Dermatology University of California, San Francisco, CA
4/2002 -	Henry Bachrach Distinguished Professor and Investigator Cardiovascular Research Institute University of California, San Francisco, CA

Medical Licensure and Board Certification:

California Medical License (G86455, issued 3/29/2002)
Missouri Medical License (R3J65, 2/1/1989 – 1/31/2004)
Diplomate, American Board of Dermatology, 1991; recertified, 2001

Honors and Awards:

Phi Beta Kappa, University of Chicago, 1979
Medical Alumni Prize for an Outstanding Oral Presentation of Research Performed During Medical School, Pritzker School of Medicine, University of Chicago, 1987
Merck/American Federation for Clinical Research Foundation M.D., Ph.D. Postdoctoral Fellowship Award, 1992
Henry Christian Award for Excellence in Research, Clinical Research Meeting, Washington, DC, May, 1993

Editorial Responsibilities:

Member, Editorial board, Genome Research, 1995 - present
Communicating Editor, Human Mutation, 2001 - present
Member, Editorial Board, Current Pharmacogenomics, 2002 - present
Member, Editorial Board, Human Genomics, 2002 – present
Member, Editorial Board, Human Molecular Genetics, term begins January 2003
Reviewer for Science, Nature, Nature Genetics, Nature Biotechnology, Genome Research, Genomics, Human Genetics, Nucleic Acids Research, and others

Professional Activities:

External Reviewer, Genome Canada, 2001-2002
External Reviewer, Hong Kong Innovation and Technology Fund, 2001-2002
Member, Genome Study Section, CSR, NIH, 1998 - 2001
Ad Hoc member of NIEHS site visits, 1998 – 2001
Member, Scientific Committee, Institute of Biomedical Sciences, Academia Sinica, Taiwan,

2002 - present

Professional Societies and Organizations:

American Association for the Advancement of Science
American Society of Human Genetics
American Academy of Dermatology
Society of Investigative Dermatology
Dermatology Foundation Leaders Society

National and International Meetings and Workshops (last 2 years):

Co-Organizer, Genome Sequencing and Biology Meeting. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, May 2000 - May 2002
Member, HGM2002Scientific Programme Committee. Shanghai, China, April 2002
Organizer, Workshop on SNP marker genotyping technologies at the American Society of Human Genetics Annual Meeting. Philadelphia, PA, October 2000
Co-Organizer, Third International Meeting on Single Nucleotide Polymorphism and Complex Genome Analysis. Taos, NM, September 2000
Member, HGM2003 Scientific Programme Committee. Cancun, Mexico, April 2003

Current Consulting Relationships and Board Memberships:

Member, Scientific Advisory Board, Paternity Testing Corporation, Columbia, MO	1997-present
Consultant, PerkinElmer, Inc. Boston, MA	1998-present
Member, Scientific Advisory Board, Quantum Dot Corporation, Hayward, CA	1998-present
Consultant, Exelixis Pharmaceuticals, Inc., South San Francisco, CA	1999-present
Member, Genome Scientific Advisory Board, Specialty Laboratories, Santa Monica, CA	2000-present
Member, Scientific Advisory Board, Vita Genomics. Taipei, Taiwan	2001-present
Member, Scientific Advisory Board, International Genomnics, Ann Arbor, MI	2002-present
Member, Scientific Advisory Board, ParAllele Genomics South San Francisco, CA	2002-present
Member, Scientific Advisory Board, Omicia, Inc. Oakland, CA	2002-present
Member, Scientific Advisory Board, FreshGene, Inc. Concord, CA	2002-present

Research Support:

Past Governmental Support (last 3 years):

Principal Investigator, NIH RO1-HG01439
High density genetic map of Xq25-Xq28
1996-1999 Total direct cost: $589,000

Principal Investigator, NSF SBR-9610342
Developing genetic markers informative in all populations
1997-1999 Total direct cost: $63,000

Principal Investigator, NIH RO1-HG01720
Technologies for diallelic marker discovery and testing
1997-2000 Total direct cost: $1,550,000

Program Director, NIH T32-AR07284
Dermatology training grant
1978-2001 Yearly direct cost: $181,484

Principal Investigator, NIH RO1-EY12557
New methods for high throughput genome analysis
1998-2002 Total direct cost: $374,840

Principal Investigator, NIH RO1-AG16869
Method for global and targeted discovery of SNP markers
1999-2002 Total direct cost: $441,000

Past Non-governmental Support (last 3 years):

Principal Investigator, Merck Genome Research Institute #176
New methods for high throughput genome analysis
1998-1999 Total direct cost: $65,000

Principal Investigator, The SNP Consortium
Allele frequency study
2000-2001 Total direct cost: $862,867

Current Governmental Support:

Principal Investigator, NIH RO1-HG01720-04
Characterization of SNP markers
2001-2004 Total direct cost: $5,097,295

Project director of Project 1, NIH U01-GM63340-01
"Identification of polymorphisms in members of pathways regulating drug activity"
In "Functional polymorphism analysis in drug pathways" (Howard McLeod, P.I.)
2001-2005 Total direct cost (Project 1): $733,009

Clinical Title and Responsibilities:

Attending physician, Dermatology Outpatient Clinic
Attending physician, Dermatology Consult Service

Past Trainees:

Linda T. Parker, Ph.D., 12/1993-7/1996, patent agent at Hoffman & Baron, LLP, Parsippany, New Jersey; law student, Rutgers University, New Brunswick, NJ
Xiangning (Sam) Chen, Ph.D., 5/1994-6/1999, Assistant Professor, Virginia Commonwealth University, Richmond, VA
Zhijie (John) Gu, Ph.D., 11/1996-4/1999, Assistant Professor, Sidney Kimmel Cancer Center, San Diego, CA
Tony M. Hsu, M.D., 7/1999-6/2001, Resident, Division of Dermatology, Washington University, St. Louis, MO

Current Trainees:

Ming Xiao, Ph.D., 2/2001-present
Denise Lind, Ph.D., 3/2002 - present

Thesis Committees:

David Politte, Ph.D., 2000 (Washington University)
George Kan, Ph.D., 2002 (Washington University)

Patents:

Kwok, P.-Y. and Chen, X. "Methods and kits for nucleic acid analysis using fluorescence resonance energy transfer" US05,945,283, issued August 31, 1999.
Kwok, P.-Y. and Chen, X. "Method for nucleic acid analysis using fluorescence resonance energy transfer" US06,177,249, issued January 23, 2001.
Kwok, P.-Y., Chen, X., and Levine, L. "Fluorescence polarization in nucleic acid analysis." US06,180,408, issued January 30, 2001.
Kwok, P.-Y., Chen, X., and Levine, L. "Fluorescence polarization in nucleic acid analysis." US06,440,707, issued August 27, 2002.

Invited Lectures (selected from the last 3 years):

1. Mutation Detection '99 International Workshop, Vicoforte, Italy, May 1999
2. Second International Meeting on Single Nucleotide Polymorphism and Complex Genome Analysis, Schloss Hohenkammer, Germany, September 1999
3. 2nd Research Symposium on the Genetics of Diabetes, San Jose, CA, October 1999
4. PE Biosystems Sixth Annual Linkage Symposium, San Francisco, CA, October 1999
5. University of Pennsylvania, Philadelphia, PA, January 2000
6. Columbia University, New York, NY, May 2000
7. IBC Annual Pharmacogenomics, SNPs and Genetic Patenting Conference, Princeton, NJ, May 2000
8. DNA 2000 International Symposium on the State-of-the-Art in Genetic Analysis, Boston, MA, June 2000
9. National Institute for Aging, Baltimore, MD, July 2000
10. DeCODE Genetics, Reykjavik, Iceland, August 2000
11. Third International Meeting on Single Nucleotide Polymorphism and Complex Genome Analysis, Taos, New Mexico, September 2000

12. SNP Genotyping Technologies in the New Millennium Workshop, ASHG Annual Meeting, Philadelphia, PA, October 2000
13. National Institutes of Health, Bethesda, MD, October 2000
14. Johns Hopkins University, Baltimore, MD, December 2000
15. University of Louisville School of Medicine, Louisville, KY, January 2001
16. Duke University, Durham, NC, February 2001
17. Arthritis Foundation "State-of-the-art" Research Research Conference, San Diego, CA, March 2001
18. The Brazilian International Genome Conference, Rio de Janeiro, Brazil, March 2001
19. University of California - San Francisco, San Francisco, CA, April 2001
20. 6th HUGO Mutation Detection Workshop, Bled, Slovenia, May 2001
21. Biogen, Cambridge, MA, May 2001
22. Pharmacia Technology Seminar, St. Louis, MO, June 2001
23. University of California – San Francisco Dermatology Grand Rounds, San Francisco, CA, July 2001
24. SCBA International Symposium, Taipei, Taiwan, August 2001
25. University of Tartu, Tartu, Estonia, September 2001
26. Fifth International Workshop on the Pharmacodynamics of Anti-Cancer Agents, Sea Island, GA, September 2001
27. Hospital for Sick Children, Toronto, ON, Canada, October 2001
28. World Congress of Psychiatric Genetics 2001, St. Louis, MO, October 2001
29. Oregon Health Sciences University, Portland, OR, November 2001
30. Emory University, Atlanta, GA, December 2001
31. University of Chicago, Chicago, IL, March 2001
32. American Association of Cancer Research Annual Meeting, San Francisco, CA, April 2002
33. HGM2002 Satellite Symposium, Hong Kong, April 2002
34. Human Genome Organization HGM2002Meeting. Shanghai, China, April 2002
35. 6th International Psoriasis Genetics Committee Meeting, Nice, France, April 2002
36. University of Michigan, Ann Arbor, MI, May 2002
37. Endocrine Society Annual Meeting, San Francisco, CA, June 2002
38. 4th Australian Workshop on Mutation Detection, Adelaide, Australia, July 2002
39. Stanford University Genome Technology Center, Stanford, CA, July 2002
40. Molecular Medicine 2002, Reykjavik, Iceland, August 2002
41. Stanford University Symposium on Genetic Studies of Human Disease, Stanford, CA, September, 2002
42. American Society of Human Genetics Annual Meeting, Baltimore, MD, October 2002
43. Institute of Biomedical Sciences, Academia Sinica, Symposium, Taipei, Taiwan, December 2002

Bibliography

(Peer-reviewed articles):

1. Kwok P-Y, Stock, LM, and Wright, TL: Partial rate factors for the thallation of toluene. J. Org. Chem. 1979;44:2309.
2. Fried, J, Kwok, P-Y, and Muellner, FW: Synthesis and enzymatic studies of fluorinated arachidonic acids. Advances in Prostaglandin, Thromboxane, and Leukotriene Research 1987;17B:803-810.
3. Kwok, P-Y, Muellner, FW, Chen, C-K, and Fried, J: Total synthesis of 7,7-, 10,10-, and 13,13-Difluoroarachidonic acids. J. Am. Chem. Soc. 1987;109:3684-3692.
4. Kwok, P-Y, Muellner, FW, and Fried, J: Enzymatic conversions of 10,10-Difluoroarachidonic acid with PGH synthase and soybean lipoxygenase. J. Am. Chem. Soc. 1987;109:3692-3698.
5. Kwok, P-Y, Gremaud, MF, Nickerson, DA, Hood, L, and Olson, MV: Automatable screening of yeast artificial-chromosome libraries based on the oligonucleotide-ligation assay. Genomics 1992;13:935-941.
6. Kwok, P-Y, Carlson, C, Yager, T, Ankener, W, and Nickerson, DA: Comparative analysis of human DNA variations by fluorescence-based sequencing of PCR products. Genomics 1994;23:138-144.
7. Gnirke, A, Iadonato, S, Kwok, P-Y, and Olson, MV: Physical calibration of yeast-artificial-chromosome based genome maps by RecA-assisted restriction endonuclease (RARE) cleavage. Genomics 1994;24:199-210.
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9. Kwok, P-Y, Deng, Q, Zakeri, H, and Nickerson, DA: Increasing the information content of STS-based genome maps: identifying polymorphisms in mapped STSs. Genomics 1996;31:123-126.
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11. Chen, X and Kwok, P-Y: Template-directed dye-terminator incorporation (TDI) assay: a homogeneous DNA diagnostic method based on fluorescence energy transfer. Nucleic Acids Res. 1997;25:347-353.
12. Chen, X, Zehnbauer, B, Gnirke, A and Kwok, P-Y: Fluorescence energy transfer detection as a homogeneous DNA diagnostic method. Proc. Natl. Acad. Sci. USA. 1997;94:10756-10761.
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14. Pulai, JI, Latour, MA, Kwok, P-Y, and Schonfeld, G: Diabetes mellitus in a new kindred with familial hypobetalipoproteinemia and an apolipoprotein B truncation (apoB-55). Atherosclerosis 1998;136:289-295.
15. Chen, X, Livak, K, and Kwok, P-Y: A homogeneous, ligase-mediated DNA diagnostic test. Genome Res. 1998;8:549-556.
16. Taillon-Miller, P, Gu, Z, Li, Q, Hillier, L, and Kwok, P-Y: Overlapping genomic sequences: a treasure trove of single nucleotide polymorphisms. Genome Res. 1998;8:748-754.
17. Zakeri, H, Amparo, G, Chen, S-M, Spurgeon, S, and Kwok, P-Y: Peak height pattern in dRhodamine and BigDye terminator sequencing. BioTechniques 1998; 35:406-414.
18. Pulai, JI, Zakeri, H, Kwok, P-Y, Kim, JH, Wu, J, and Schonfeld, G: Donor splice mutation (665+1 G_T) in familial hypobetalipoproteinemia with no detectable apoB truncation. Amer. Journ. Med. Gen. 1998;80:218-220.

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20. Chen, X, Levine, L, and Kwok, P-Y: Fluorescence polarization in homogeneous nucleic acid analysis. Genome Res. 1999;9:492-498.
21. Taillon-Miller, P and Kwok, P-Y: Efficient approach to unique single nucleotide polymorphism discovery. Genome Res. 1999;9:499-505.
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32. Marth, G, Yeh, R, Minton, M, Donaldson, R, Li, Q, Duan, S, Davenport, R, Miller, RD, and Kwok, P-Y: Single nucleotide polymorphisms in the public database: how useful are they? Nature Genetics 2001; 27:371-372.
33. Collins, A, Ennis, S, Taillon-Miller, P, Kwok, P-Y, Morton, NE: Allelic association with SNPs: Metrics, populations, and the linkage disequilibrium map. Hum Mutat. 2001; 17:255-262.
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38. Vieux, EF, Kwok, P-Y, and Miller, RD. Primer Design for PCR and Sequencing in High Throughput Analysis of SNPs. BioTechniques 2002, Suppl:28-30, 32.
39. Speckman, RA, Daw, J, Helms, C, Duan, S, Cao, L, Taillon-Miller, P, Kwok, P-Y, Menter, A, and Bowcock AM. Novel immunoglobulin superfamily gene cluster mapping to a region of human chromosome 17q25 linked to psoriasis susceptibility. Human Genetics, in press.

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(Invited articles):

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EXHIBIT
2
PENGAD 800-631-6989

BioTeZ Berlin Buch GmbH
Biochemisch - Technologisches Zentrum

BioTeZ Berlin Buch GmbH • Robert-Rössle-Straße 10 • 13125 Berlin

To whom it concern

1) I am the inventor and owner of the patent:

Strohner, P. Immer, U.: Surfaces coated with Streptavidin/Avidin, US-Patent 6,270,983 (7th August 2001), European patent pending. Positive evaluted by European Patent Office.

on means and methods for coating solid surfaces with streptavidin for various downstream applications, including DNA analysis. I am also the founder/owner of the company Biotez, which for many years has had its major business activity in coating different types of solid monolayer surfaces with streptavidin. In this capacity, I offer the following Expert Declaration on issues pertaining to the physical properties of streptavidin-coating of solid surface and molecules bound to such surfaces through biotin-streptavidin interaction.

2. Regardless of coating procedure details, immobilization of streptavidin onto solid-surfaces (such as plastic microtiter plates and membranes) will result in a reactive streptavidin monolayer. DNA molecules which are bound to this reactive streptavidin monolayer will inevitably form a superimposed DNA monolayer

3. The term 'monolayer' indicates a molecular arrangement wherein a number of DNA molecules are all similarly aligned, all at approximately the same distance from the basal binding surface, and all available for chemical reactions (e.g., hybridization) with other molecules that may approach from one and the same direction only.

4. DNA monolayer structures have distinct physicochemical properties and low binding capacities (whether created by streptavidin-biotin linkages or any of a wide range of chemical bond arrangements) that are fundamentally different to those observed for DNA in solution or immobilized in 3-D arrangements in a gel matrix. Unlike in solution and in gels, reacting species cannot approach a DNA monolayer immobilized to a solid surface from all-around in 3-D space in an unhindered manner. DNA monolayers may also have increased target-target interactions and reduced binding/reaction capacity relative to DNA in solution or in gels.

5. In conclusion, it is my understanding that DNA bound to a solid surface by a streptavidin-biotin link would be considered in the field of DNA hybridization to be a monolayer of DNA molecules, with all that entails.

6. I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signature

Dr. Pavel Strohner

CEO

Date: 27th Nov. 2003


EXHIBIT
3
PENGAD 800-631-6989

Docket Number: 40225.000

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	:	Brookes, et al.
Serial No.	:	09/755,747
Examiner	:	Jeffrey Fredman
Art Unit	:	1637
Filed	:	5 January 2001
For	:	DETECTION OF NUCLEIC ACID POLYMORPHISM

DECLARATION OF JOHN D. BALDESCHWIELER

1. I am a Professor of Chemistry at Caltech, and for many years I have researched into areas such as spectroscopy, microscopy, and the application of these and other molecular techniques to the study of biological systems. In that capacity, I offer the following Expert Declaration on issues pertaining to a 1995 paper on which I was the senior investigator (Real-time detection of DNA hybridization and melting on oligonucleotide arrays by using optical wave guides. Stimpson DI, Hoijer JV, Hsieh WT, Jou C, Gordon J, Theriault T, Gamble R, Baldeschwieler JD. Proc Natl Acad Sci U S A. 1995 Jul 3;92(14):6379-83: 'Stimpson et al').

2. Specifically, the purpose of the stated publication was to establish a means for real-time tracking of DNA melting that would work effectively on a solid surface. Both before and after the publication of Stimpson et al, , one skilled in the art would not expect the DNA binding capacity of any of the stable and common 2-D surfaces and chemistries to yield sufficiently strong fluorescent signals sufficiently 'instantly' (sub-second) in a fluorescence based assay method to allow for dynamic tracking of signal changes in real-time, when applying practically useful rates of heating. One skilled in the art would, therefore, most rationally turn to 3-D (gel-type) arrays to solve this widely recognized problem, since the considerable 3rd dimension provides far greater capacity and scope for DNA binding and manipulation.

3. The known limitations of solid surface fluorescence assays (i.e. 2D surfaces) compared to gel type alternatives (i.e. 3D matrices), are repeated emphasized in the Introduction, Results, and Discussion sections of Stimpson et al . Indeed, these factors were a large part of the motivating force that led us explore the alternative signal generation mechanism that we describe in Stimpson et al. Our solution was to channel an evanescent wave of intense light through a wave-guide in such a way that it became scattered (and thereby externally detectable) only from those surface regions to which suitably modified duplex DNA strands were bound.

4. Given the well-known problems with fluorescence-based assays on a solid surface, a skilled person in the field would not have been motivated to replace the signal generation mechanism described in Stimpson et al with any kind of fluorescence-based system.

5. I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Signature... John D Baldeschwieler Date.... 11/11/03

John D. Baldeschwieler
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EXHIBIT
4
PENGAD 800-631-6989

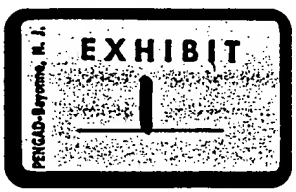
A Monolayer of Covalently Coupled Streptavidin

Analysis and close calculations show that the bead-coating consists of a monolayer of covalently coupled streptavidin, presenting a perfect environment for the binding of biotinylated targets. As opposed to multilayers of streptavidin, a monolayer does not introduce the risk of streptavidin being sterically hidden and un-available for binding of your ligand/target. The absence of excess physically adsorbed streptavidin ensures superior reproducibility and low batch-to-batch variations. The monolayer on Dynabeads® Streptavidin also secure that only neglectible amount of streptavidin will have the possibility to leak (<0.2% of total streptavidin attached after 2 months at 37°C).

There are three different streptavidin-coated Dynabeads® available from Dynal Biotech.

- Dynabeads® M-280 Streptavidin (Prod. No 112.05/06 and 602.10) is the product most frequently chosen.
- The more hydrophilic Dynabeads® M-270 Streptavidin (Prod. No 353.02/13) is suitable for nucleic acid applications with extreme demands, and has a more negative charged surface at pH =7.
- Dynabeads® MyOne™ Streptavidin (Prod.No. 650.01, 650.02 and 650.03) are similar to the Dynabeads® M-270 Streptavidin in their degree of hydrophilicity. But in contrast to the two 2.8 µm beads mentioned above, the MyOne™ beads are one micron in diameter. The Dynabeads® MyOne™ Streptavidin have a high binding capacity and superior performance in automated systems, and are optimal for IVD assays.

All Dynabeads® show excellent results in reproducibility and stability.



SAM2® Biotin Capture Membrane

Promega

Technical Bulletin No. 547

INSTRUCTIONS FOR USE OF PRODUCTS V2861 AND V7861. *PLEASE DISCARD PREVIOUS VERSIONS.*
All technical literature is available on the Internet at www.promega.com
Please visit the web site to verify that you are using the most current version of this Technical Bulletin.

I. Description

The SAM2® Biotin Capture Membrane(a) binds biotinylated molecules based on their affinity for streptavidin. The proprietary process by which the SAM2® Membrane is produced results in a high density of streptavidin on the filter, providing rapid, quantitative substrate binding in the nmol/cm^2 range. In addition, the Membrane has been optimized for low nonspecific binding. Figure 1 outlines the procedure for use of the SAM2® Biotin Capture Membrane with biotinylated substrate molecules as used in Promega's SignaTECT® Protein Kinase Assay Systems(a).

The Membrane is available either as a large, prenumbered, partially cut sheet (approximately 10.5 x 15.0cm; Cat.# V2861) or as a smaller, uncut sheet (approximately 7.6 x 10.9cm; Cat.# V7861). The partially cut Membrane (Cat.# V2861) allows easy separation into 96 individual squares and is designed for small-scale experiments where high binding capacity is required. The uncut sheet (Cat.# V7861) can be analyzed as a whole Membrane or may be cut into the size desired. The uncut Membrane allows for sample application using a multichannel pipettor. Both Membranes may be analyzed using phosphorimaging, autoradiography or scintillation counting to quantitate results. The Membranes have also been used successfully with chemiluminescence detection techniques. The use of fluorescence for detection of captured molecules is not recommended at this time.

The SAM2® Membrane, used as recommended in this technical bulletin, provides a number of advantages over other commercially available streptavidin products. These advantages include:

- **Versatility:** Analysis of biotinylated substrates can be applied to a wide variety of substrate types without the need to optimize each substrate for binding to a matrix. Available in 96-square (partially cut) and solid-sheet (uncut) formats, the user can perform experiments with a wide array of sample numbers and sizes without changing the analysis technique.

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- **Specificity:** The combination of protein denaturant and high salt washes minimizes nonspecific binding to the Membrane without interfering with the high affinity interaction between streptavidin and biotin.
- **High Signal-to-Noise Ratios:** The stringent washing conditions employed assist in attaining very low background counts.

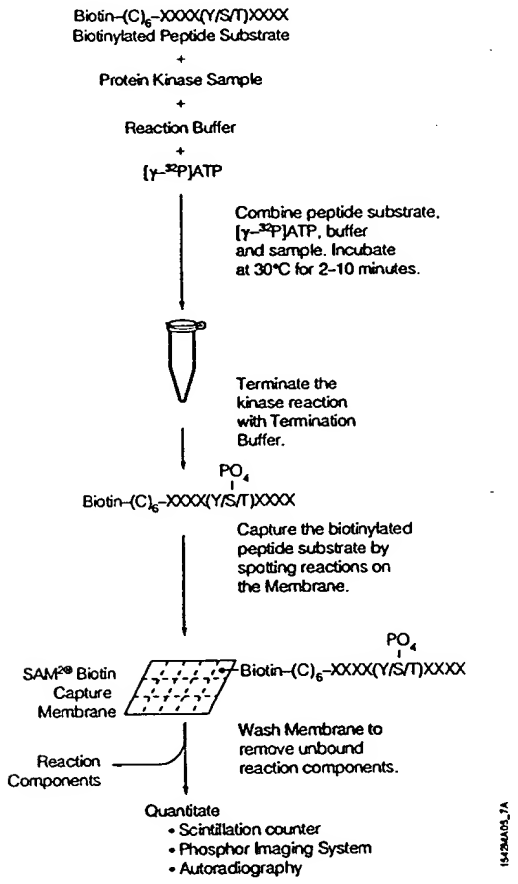

Figure 1. Flow diagram depicting use of the SAM2® Biotin Capture Membrane for analysis of kinase activity as used in Promega's SignaTECT® Protein Kinase Assay Systems.

II. Product Components

Product	Size	Cat.#
SAM2® Biotin Capture Membrane	96 samples	V2861
SAM2® Biotin Capture Membrane (uncut)	7.6 x 10.9cm	V7861

Storage Conditions: Store the SAM2® Biotin Capture Membrane in the resealable bag. The Membrane is stable for up to 6 months at –20°C and can be stored at 4°C for up to four weeks.

III. SAM2® Biotin Capture Membrane Background and Characteristics

A. Background

It is frequently desirable in molecular biology and enzymatic analysis to separate a specific substrate from other compounds in a reaction mix. This separation is usually accomplished using a solid matrix that selectively binds the substrate. The matrices, which are often based on ionic or metal ion interactions, are only grossly selective and thus are prone to undesirable variations in performance when different substrates, enzymes or washing conditions are used (1,2). Potential problems include variations in: 1) background and signal intensities depending upon the degree of washing; 2) binding affinity of the substrate based on composition; 3) substrate specificity due to alteration of the substrate to achieve efficient matrix binding. In addition, variations in performance with standard matrices can occur due to the detection of signal from miscellaneous substrates present in complex samples such as crude cell or tissue extracts, which can bind nonspecifically to the matrix (2,3).

Some assays have circumvented these problems by using the high-affinity streptavidin:biotin interaction ($K_d = 10^{-15}M$) to separate the substrate from other reactants (4). This has been accomplished using biotinylated substrates and streptavidin-coated plates or beads. Unfortunately, the limited capacity of streptavidin-coated plates and streptavidin-coated beads places restrictions on the parameters of the assay, thereby limiting the utility of these formats. For example, many enzymes, particularly protein tyrosine kinases, have high K_m values for peptide substrates, frequently above 25µM and as high as 1mM (5). To work at maximal sensitivity (near V_{max}) the peptide substrate concentrations must be at least 3 times greater than the K_m. The binding capacity of commercially available streptavidin-coated plates and beads is generally at least one order of magnitude below this desired capacity. Even if the enzyme activity were sufficiently high to allow suboptimal substrate concentrations to be used at the 96 sample level, further restrictions would be encountered when using miniaturization to 384 samples or higher density arrays.

B. Membrane Characteristics

The SAM2® Membrane overcomes the problems described above by providing the binding capacity to work at optimal conditions at the 96 sample level while retaining sufficient signal-to-noise ratios to allow miniaturization to higher sample density arrays. The binding of biotin to streptavidin is rapid and strong; binding of the biotinylated molecules to the SAM2® Membrane occurs within 30 seconds of sample application. Once formed, this association is unaffected by extremes in pH, temperature, organic solvents, ionic and nonionic detergents and denaturing agents (Table 1; 4).

The 96-square-sheet format (Cat.# V2861) is prenumbered and partially cut so that individual squares can be easily identified, separated and placed into scintillation vials or left intact and quantitated by phosphorimaging or by conventional autoradiography. The uncut sheet (Cat.# V7861) can be utilized for multiple samples in the solid-sheet format, or it can be custom cut to accommodate various sample size and sample number specifications.

Promega

Table 1. Stability Data for the SAM2® Biotin Capture Membrane*.

Factor	Range Compatible with the SAM2® Membrane
Organic solvents	95% ethanol
Detergents	1% SDS, 1% Chaps, 1% Triton® X-100, 1% Tween® 20, 1% Tween® 40
Denaturing agents	5M guanidine hydrochloride, 2M urea
pH	2.0–10.0
Ionic strength	0–5M NaCl
Binding of streptavidin:biotin	$K_d = 10^{-15}$M
Binding time	<30 seconds
Background counts	0.02–0.1%

*See reference 6

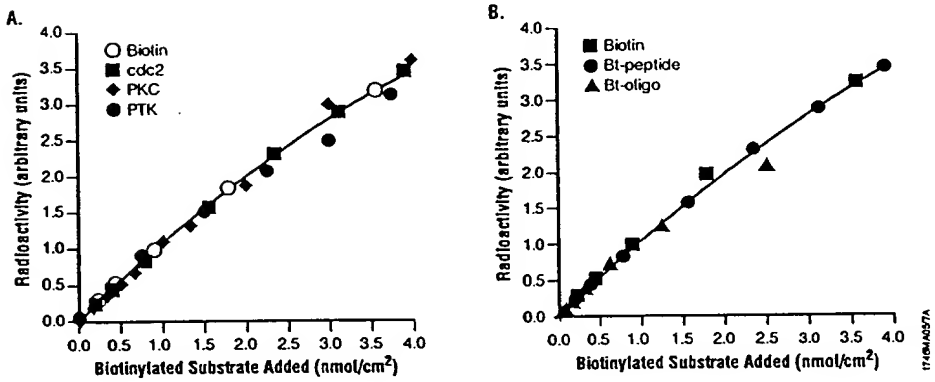

Figure 2. Binding of biotin and various biotinylated substrates to the SAM2® Biotin Capture Membrane. Panel A: Binding of several different biotinylated peptide substrates and biotin alone to the SAM2® Membrane was compared. The indicated amounts (x-axis) of radioactive biotin and biotinylated peptide substrates for cdc2, PKC and PTK in 2.5M guanidine-HCl, were spotted onto the SAM2® Membrane, washed 4X in 2M NaCl, 4X in 2M NaCl plus 1% H_3PO_4 and 2X in water, were dried and the results quantitated by scintillation counting. **Panel B:** Radioactive biotin and biotinylated peptide (Bt-peptide) were spotted onto the Membrane and washed as stated above. Radioactive, biotinylated oligonucleotide (Bt-oligo) in water was spotted onto the SAM2® Membrane, washed 4X in 1% SDS, 2X in water, 4X in 2M NaCl and 2X in water, dried and the results quantitated by scintillation counting.

Studies performed in our laboratories have tested the SAM²® Membrane with many different biotinylated peptides and oligonucleotides. We have shown that the binding affinity of the Membrane for these substrates is similar to the affinity of the Membrane for biotin alone (1–3). In addition, binding of biotinylated molecules to the SAM²® Membrane occurs independently of amino acid or molecular sequence (Figure 2). This property allows the comparison of multiple peptide substrates, which is especially important for those peptide substrates that do not bind well to standard matrices (7–9).

IV. Procedure for Use of SAM²® Biotin Capture Membrane in Kinase Assays

The following procedure is recommended for use of the SAM²® Membrane in kinase assays with biotinylated peptide substrates. Please note that you will need to optimize the buffers and washing protocol for use of the Membrane with other types of molecules.

1. Wearing gloves, cut (using scissors or a razor blade) the required number of squares from the partially cut SAM²® Membrane. Alternatively, the squares may remain connected as a sheet to minimize handling. When working with the uncut Membrane (Cat.# V7861), either cut into individual pieces or handle as a whole sheet. Return any unused Membrane to the resealable bag at 4°C or –20°C.

2. After completion of the protein kinase reactions, terminate with 0.5 volume of 7.5M guanidine hydrochloride solution in water (final concentration of 2.5M guanidine hydrochloride).

3. When using the partially cut Membrane (Cat.# V2861), apply 0.1–25µl of the terminated kinase reaction (≤2nmol of peptide) to an individual Membrane square or apply a maximum of 15µl per square if the squares are still connected. For the uncut Membrane (Cat.# V7861), apply a substrate concentration of ≤1.3nmol/cm² (see Note below). If applying samples with a multichannel pipettor, the maximum volume applied should be ≤5µl. Allow the samples to absorb to the Membrane; there is no need to dry the Membrane completely before washing.

   Note: It is possible to bind more than 1.3nmol/cm² and retain a linear binding response. The linear binding response above 1.3nmol/cm² will depend upon the assay being performed and must be determined by the user (Figure 2).

4. Place the SAM²® Membrane squares or the intact sheet containing samples into a washing container. Wash, using a minimum of 100ml of each solution, changing solutions after each wash. Using an orbital platform shaker set on low speed or by manual shaking, follow this washing procedure:

   Wash 1 time for 30 seconds with 2M NaCl.
   ↓
   Wash 3 times for 2 minutes each with 2M NaCl.
   ↓
   Wash 4 times for 2 minutes each with 2M NaCl in 1% H_3PO_4.
   ↓
   Wash 2 times for 30 seconds each with deionized water.

   Total wash time <20 minutes.

Do not exceed 30µl per square.

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Notes:

If using radioisotopes, dispose of the radioactive wash solution in accordance with the regulations of your institution.

More or less washing may be appropriate to achieve acceptably low background counts; this should be determined empirically.

For rapid drying, a final 15-second, 95% ethanol wash can be used. Longer washes with ethanol may cause the ink to run slightly.

5. Dry the SAM2® Membrane squares on a piece of aluminum foil under a heat lamp for 5–10 minutes or air-dry at room temperature 30–60 minutes. (If the SAM2® Membrane has been washed with ethanol, shorten the drying time to 2–5 minutes under a heat lamp or 10–15 minutes at room temperature.)

6. **Analysis by Scintillation Counting:** If you are using radioisotopes and the SAM2® Membrane (Cat.# V2861) is still intact, separate the squares using forceps, scissors or a razor blade and place into individual scintillation vials. Add scintillation fluid to the vials and count. The uncut Membrane (Cat.# V7861) may be cut into sample pieces and each piece analyzed in individual vials after addition of scintillation cocktail.

   Analysis by Phosphorimaging: Alternatively, the SAM2® Membrane may remain intact and the intact SAM2® Membrane may be analyzed using a phosphorimaging system.

V. Related Products

Product	Size	Cat.#
SAM2® 96 Biotin Capture Plate[a]	96 well plate	V7541
	5 x 96 well plates	V7542
SignaTECT® Protein Tyrosine Kinase Assay System[a]	96 reactions	V6480
SignaTECT® Protein Kinase C (PKC) Assay System[a]	96 reactions	V7470
SignaTECT® cAMP-Dependent Protein Kinase (PKA) Assay System[a]	96 reactions	V7480
SignaTECT® DNA-Dependent Protein Kinase Assay System[a]	96 reactions	V7870
SignaTECT® Calcium/Calmodulin-Dependent Protein Kinase (CaMKII) Assay System[a]	96 reactions	V8161
SignaTECT® cdc2 Protein Kinase Assay System[a]	96 reactions	V6430
PepTag® Non-Radioactive PKC Assay[b]	120 reactions	V5330
PepTag® Non-Radioactive cAMP-Dependent Protein Kinase Assay[b]	120 reactions	V5340

VI. References

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9. Schaefer, E. et al. (1996) Detection and Quantitation of Protein Tyrosine Kinases *Promega Notes* **59**, 2.

[a]U.S. Pat. No. 6,066,462 has been issued to Promega Corporation for quantitation of protein kinase activity.

[b]U.S. Pat. No. 5,580,747 has been issued to Promega Corporation for a non-radioactive enzyme assay.

PepTag, SAM² and SignaTECT are trademarks of Promega Corporation and are registered with the U.S. Patent and Trademark Office.

Triton is a registered trademark of Union Carbide Chemicals and Plastics Co, Inc. Tween is a registered trademark of ICI Americas, Inc.

All prices and specifications are subject to change without prior notice.

Product claims are subject to change. Please contact Promega Technical Services or access the Promega online catalog for the most up-to-date information on Promega products.

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Acronyms & Abbreviations (most common first) Wednesday, June 30, 2004

AF Acronym Finder
home/new search

Option: exact acronym

New acronym search: SAM Find

What does **SAM** stand for?

Your search returned 114 meanings

Search again sorting by... Most Common | Information Technology | Military & Government | Science & Medicine | Organizations, Schools, etc. | Business & Finance | Slang & Chat | Alternative Searches

Acronym	Definition	@Amazon.com
SAM	Secure Access Module	
SAM	Security Account Manager	
SAM	Security Authentication Module	
SAM	Segment Access Manager	
SAM	Self Assembling Material	
SAM	Self Automated Machine	
SAM	Self-Assembled Monolayer	
SAM	Semi-Analytic Model (galactic modeling)	
SAM	Sensor Arrays and Multichannel Signal Processing	
SAM	Serbia and Montenegro	
SAM	Serial Access Memory	
SAM	Service Access Multiplexer (Bellcore)	
SAM	Service Account Manager	
SAM	Service Adaptation Module (Tachion)	
SAM	Service Assurance Manager (Smarts)	
SAM	Service Automation Module (Opsware)	
SAM	Servicing And Maintenance	
SAM	Serving Area Multiplex Sites	

EXHIBIT 3

Image FlashPlate Streptavidin coated, 40 plates

Product categories:
Select a product category

Product No.	Product Name	Unit Size	Unit Price	
RMP110	Image FlashPlate Streptavidin coated, 40 plates	1/PK	*Log In or Register	Add to Order

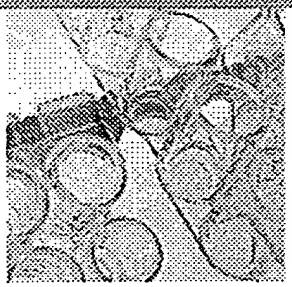
*U.S. and Canadian customers please log in or register to see price.

Product Information

Description:

Basic Image FlashPlate pre-coated with Streptavidin and a blocking protein. For capture of biotinylated molecules. Widely used for many types of enzyme assays.

EXHIBIT 4



FEATURES
DETAILS
ACCESSORIES
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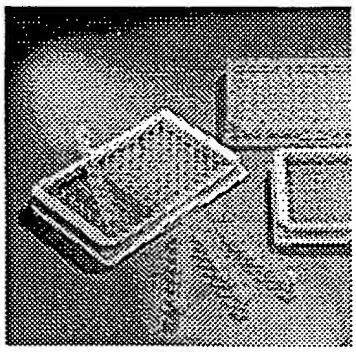
Streptavidin Coated Plates

Streptavidin Coated Plates



- Ideal for binding of biotinylated biomolecules such as peptides, antibodies, oligonucleotides or haptens
- Streptavidin coated area of 154 mm² (area covered by a volume of 200 µl)
- Binding capacity for biotin of at least 13 pmol/well*
- Stable at room temperature
- General coating protocol available on request
- Available in C96 Plates or C8 well strips
- Other formats available on request

* Depending on size or sterical properties of a given biomolecule the actual binding capacity might be different.

Details

Streptavidin Coated Plates
Polystyrene, 96 wells
External dimensions 128 x 86 mm

Cat. No.	236001	236004
Configuration	C96	C8
Colour	Transparent	Transparent
Total volume, µl/well	350	350
Coated well volume, µl	200	200
Units per pack/case	1/15	1/15
Availability	Not Available	Not Available

Accessories

Accessories for Streptavidin Coated Plates

Cat. No.	430805	430082
Description	8 Well strip cap	8 Well strip cap
Material	Polyethylene	Polyethylene
Sterile	+	-
Units per pack/case	12/120	12/120
Availability	Not Available	Not Available

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EXHIBIT 5

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27695 25292 24954 24765 23945

Tested Applications: enzyme assay

Application Notes:
For use in plate assays.

EXHIBIT 6

For general laboratory.
FOR *IN VITRO* USE ONLY.

StreptaWell*

Instruction Manual

Version 1, January 2003

Store the plates at 2–8°C

	StreptaWell*	StreptaWell High Bind*
Plate Type	**Cat. No. (pack size)**	**Cat. No. (pack size)**
96-wells, transparent, C-bottom	**1 734 776** (15 Plates)	**1 989 685** (15 Plates)
12 × 8-well strips and frame, transparent, C-bottom	**1 664 778** (5 Plates)	**1 645 692** (5 Plates)
12 × 8-well strips and frame, transparent, nuclease-free, C-bottom	**1 768 000** (5 Plates)	
384-wells, transparent, C-bottom	**1 989 669** (5 Plates)	
96-wells, white, C-bottom	**1 989 707** (15 Plates)	**1 989 693** (15 Plates)
12 × 8-well strips and frame, white, C-bottom	**1 602 861** (5 Plates)	**1 989 715** (5 Plates)
384-wells, white, C-bottom	**1 989 677** (5 Plates)	
96-wells, black, C-bottom	**1 734 784** (15 Plates)	
12 × 8-well strips and frame, black, C-bottom	**1 602 837** (5 Plates)	

*covered by EP Patent EP-B 0269092 and US Patent US 5,061,640 granted to Roche Applied Science

Roche

1. Preface

2. Introduction

2.1 Product overview

StreptaWell

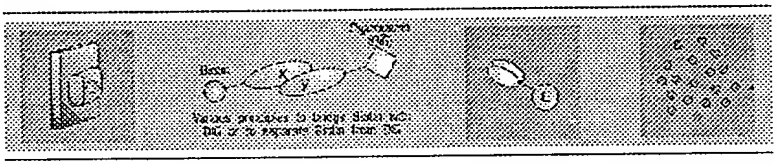
Streptavidin-coated microplates are available in two binding capacities:

- **StreptaWell** (regular binding capacity) and
- **StreptaWell, High Bind** (high binding capacity).

The different binding capacities result from different coating procedures using different starting materials.

Streptavidin-based assay system

The modular streptavidin-based assay system is composed of more than 100 different items which enable the set up of almost any biochemical assay. The system consists of universal modules and parameter-specific components (Fig.1). The universal modules are identical for most applications, comprised of a streptavidin-coated microplate (StreptaWell), a Roche Applied Science proprietary tracer system anti-digoxigenin-enzyme conjugate), and a set of different substrate alternatives to generate a chemiluminescent, fluorescent or colorimetric signal.
The parameter-specific components (which have to be designed according to the parameter of interest) are biotin-labeled for immobilization purposes, and digoxigenin (DIG)-labeled for detection and quantification. The techniques associated with labeling the parameter-specific components like peptides, proteins, and nucleic acids are well established.
Protocols, highly convenient kits, and reagents are all available from Roche Applied Science.



solid phase* StreptaWell	parameter-specific components**	secondary detection with anti-DIG-POD* or anti-DIG-AP*	chemiluminescent, fluorescent or colorimetric substrates*

* Universal Modules, available in various DIG Detection ELISAs
** Parameter-specific components, can be labeled via Nucleic Acids- and Protein-Labeling Kits, available from Roche Applied Science

Fig. 1: Principle of the Modular Streptavidin-based Screening System

Detection principles

StreptaWell can be used with the following different detection principles:

	Detection		
StreptaWell	Colorimetric	Chemiluminescent	Fluorescent
transparent	++	-	+
white	-	++	++
black	-	+	+

Application

StreptaWell can be used to study:

- Enzyme activities
- Immunoassays
- Protein modifications
- Protein-Protein interactions / Receptor Binding
- Protein-Nucleic acid binding
- Nucleic acid amplification and hybridization
- Cell products (cytokines, steroides etc.)
- Reporter genes

2.2 Product characteristics

Specifications The following table gives an overview about the coating specifications.

	StreptaWell 96	StreptaWell 96 High Bind	StreptaWell 384
SA-coated area (indicated as volume)	≥300 µl	≥300 µl	≥60 µl
Blocked volume	≥320 µl	≥ 320 µl	≥75 µl
Total biotin binding capacity* (competition assay)	≥5 ng/well ≥20 pmol/well ≥70 nM	≥20 ng/well ≥80 pmol/well ≥ 200 nM	≥1 ng/60 µl well ≥4 pmol/60 µl well ≥70 nM
Total binding capacity for biotin-labeled antibodies	1.5 µg/well	1.5 µg/well	0.3 µg/60 µl well
Coating variance between individual wells	<5%	<5%	<8%
CV between different plates	<10%	<10%	<15%
SA-leaching	<5 ng/well	<5 ng/well	<1 ng/60 µl well

* well: 300 µl

continued on next page

2.2 Product characteristics, Continued

Homogeneity and production variances	The proprietary coating process guaranties homogenous and reproducible coatings with unique features: • little variances between individual wells (intra-assay-variance) • little variances between different plates (inter-assay-variance) • high lot-to-lot reproducibility • high signal-to-noise ratio • low background • almost no leaching
Storage/ stability	The unopened plates are stable at 2-8°C through the expiration date printed on the label. ***Note***: Store dry and protected from light.
Binding capacity	The biotin binding capacity as given under specifications is identical to the number of biotin binding sites present. Depending on the size and sterical properties of a given biomolecule, the actual molar binding capacity may often be below this maximum value.
Factors influencing binding capacity	Even though the binding capacity could be reduced to some extent, the integrity of the coating and the stability of the streptavidin-biotin interaction has proven to be remarkably resistent to a variety of harsh conditions: • buffers generally used in molecular biology like SSC, TEN, RIPA, TBS, etc. • 4 M Guanidinium-thiocyanat, 1 h, 15-25°C • 4 M Urea, 37°C, 1 h • 50% formamide, 56°C, 1 h • 1% SDS, 37°C, 1 h. (Some detergents may influence the properties of StreptaWell in concentrations above 0.1 to 1%.) • pH 4-10, • elevated temperatures up to 75°C. (Do not heat above 75°C.)
Sensitivity	Potential sensitivity-limiting factors for a microplate assay are: • affinity of the specifically interacting components • sensitivity of the detection system • non-specific binding (signal-to-noise). Therefore maximum sensitivity of the detection method can only be achieved if the affinities of the interacting components are not limiting for the assay and the total of non-specific signal is well below 0.1 % of total signal.
Pigmentation	The pigmentation of the microplates is to prevent 'cross-talk' (light exchange) between individual wells. Both, the white and the black plates have cross-talk-levels well below 0.1%. Due to light-reflection the signal intensity in white plates is about 10 times higher as obtained from black plates. If the background-signal is low, this could lead to an increase in sensitivity.
Caution	Do not sonify microplates or wells.

3. Procedures and required materials

3.1 Before you begin

General

StreptaWell plates are ready to use as supplied. No extra rehydration step is needed.

Additional substrates required

In the following table you will find substrates available from Roche Applied Science suitable for the different detection principles:

	Substrates		
StreptaWell	Colorimetric	Chemiluminescent	Fluorescent
transparent	4-Nitrophenyl-phosphate CPRG ABTS BM Blue POD TMB	-	+
white	-	BM CL ELISA Substrates: (AP) (POD)	AttoPhos
black	-	+	+

3.2 Labeling of molecules

Additional reagents required	For labeling of biomolecules or biomolecule complexes, highly convenient labeling reagents or ready-to-use labeling kits are available, see Related Products.
Labeling of proteins, peptides and small molecules	Labeling of proteins, peptides and small molecules like haptens can be performed under mild conditions via free amino groups, sulfhydryl groups, disulfide bridges or oxidized sugar residues (aldehyde or keto groups). In general it is recommended to label proteins via free amino groups (lysyl residues). Labeling with biotin normally has no effect on the properties of proteins. In small molecules like haptens and oligopeptides, the label has to be conjugated to a part of the molecule, which is not essential for function. Often a spacer structure between label and hapten (or ligand) is advantageous to allow optimal interaction with antibodies (or receptors).
Labeling of nucleic acids	Nucleic acid labeling may be performed chemically or enzymatically with reagents or kits. In molecular biology, double labeling with biotin and digoxigenin is often used to analyze nucleic acids in polymerisation and hybridization experiments.

3.3 ELISA Protocol

Before you begin

- When possible, allow biotinylated components to bind to steptavidin under physiological buffer conditions. For more stringent conditions see section 3.4.
- Immobilization of biomolecules via steptavidin/biotin interaction is at least as effective as direct coating to physically activated surfaces. In many cases, e.g. small molecules, oligonucleotides or peptides, the binding of biotinylated components will be much more efficient.
- After washing out excess biotinylated substance, all further steps may follow the standard protocol as optimized for a particular parameter. Volumes and general conditions are given below.

General ELISA protocol

In the following table please find the components needed for a general ELISA procedure:

Step	Buffers	Volume	Time/Temp.(°C)
Binding of biotinylated component	PBS or TBS containing 0.1% BSA	50-100 µl	15-60 min/15-25°C or 35°C
Washing steps	PBS or TBS containing 0.1% BSA and/or further additives	300 µl (each wash cycle)	3-5 × with 5 min incubation between individual washes/ 15-25°C
Antibodies, antigen, etc. incubations	PBS or TBS containing 0.1% BSA and/or further additives, depending on the components used	100-150 µl	60 min/ 15-25°C or 35°C
Secondary detection component		200 µl	
Colorimetric-, chemiluminescence-or fluorescence substrate solutions	Prepare solutions according to the manufacturers protocol or use ready-to-use reagents	250 µl (including volume of trigger solutions, if required)	Depending on enzyme/ substrate system/ 15-25°C

3.4 Optimizing ELISA protocols

Antibody-conjugate concentration

When changing to fluorescent or chemiluminescent detection, the concentrations of the conjugates often have to be adapted. However, at the first attempt, the conjugate concentration should be used as recommended by the supplier or as optimized for a particular colorimetric assay. If the conjugate contributes to non-specific binding, its concentration may be lowered down to 1:10.

Reduction of non-specific binding

Optimizing non-specific binding might be a prerequisite for highly sensitive detection. To reduce background, either additional components may be added to washing-, incubation- and conjugate buffers and/or the concentrations of the specific interacting components may be lowered.

The following additives may be used:	Additives / Concentration
Salt	0.5-1.0 M NaCl
Complexing agent	1-5 mM EDTA
Detergent	0.05-0.1% Tween[1]20
Protein	0.1-1% BSA, serum, casein, milk powder

Washing conditions

Most interactions which contribute to non-specific binding are of low/ intermediate affinity and therefore reversible in character. Prolonged intervals between individual washes (we recommend at least 3 repeated washes) favor dissociation from non-specific binding sites.

Handling very concentrated samples

Samples exceeding the measuring range should be diluted with incubation buffer and the ELISA should be repeated. This dilution factor has to be taken into account when calculating the content.

4. Appendix

4.1 Trouble-shooting

Problem	Possible cause	Recommendation
Week or no signal		Check instrument settings.
		Check activity of marker enzyme/ molecule.
	Water ingredients that influence the test negatively.	Always use double distilled water for reconstitution and preparing the working solutions; take care that the water is not microbially contaminated!
	Interference of buffer components with substrate	Check conjugate buffer for incompatible components (e.g. NaN_3, SH-reagents).
	Inadequate incubation time and temperature	Check protocol (incubation times/ temperatures, buffer conditions, etc.) and concentrations of primary antibody or antigen.
	Substrate or vial used to aliquot substrate contaminated	Check substrate reagent for storage conditions and biological contamination. Use freshly prepared reagent.
		Check integrity of positive control.
High background signal	Washing procedure not efficient	Prolong washing procedure (number of washes, interval between washes).
	Non-specific interaction of buffer additives	Try different additives with the washing/ incubation buffers to block non-specific interactions.
	Inadequate concentration of detection component, e.g. antibodies	Modulate concentrations for detection components, e.g. primary/secondary antibody.

4.2 Related products

Product	Pack Size	Cat. No.
Streptavidin (SA)-coated and Anti-DIG-coated Tubes and Magnetic Particles		
SA-coated Tubes	4 × 25 tubes	1 602 845
SA-coated PCR Tubes (Strips)	24 strips of 8 × 0.2 ml tubes and caps	1 741 772
SA Magnetic Particles	2 ml 10 ml	1 641 778 1 641 786
Anti-Digoxigenin Magnetic Particles	2 ml	1 641 751
Magnetic Particles Separator	1 separator (for 4 × 1.5 ml tubes). 1 separator (for 3 × 15 ml or 50 ml tubes; or for a 96 well MP).	1 641 794 1 858 025
Colorimetric Substrates		
CPRG	250 mg	884 308
ABTS	2 g	102 946
ABTS Solution	3 × 100 ml	1 684 302
ABTS Tablets	20 tablets (5 mg for 5 ml)	1 204 521
ABTS Tablets	20 tablets (50 mg for 50 ml)	1 112 422
BM Blue POD Substrate, precipitating	100 ml	1 442 066
BM Blue POD Substrate, soluble	100 ml	1 484 281
TMB	1 g	784 974
4-Nitrophenylphosphate	5 g	107 905
4-Nitrophenylphosphate	10 tablets	726 923
Chemiluninescent Substrates-BM CL ELISA Substrates		
BM Chemiluminescence ELISA Substrate AP	150 ml	1 759 779
BM Chemiluminescence ELISA Substrate POD	250 ml	1 582 950
Fluorescent Substrates		
AttoPhos	for 1800 wells	1 681 982

continued on next page

4.2 Related products, Continued

Product	Pack Size	Cat. No.
Labeling proteins		
Biotin Protein Labeling Kit	1 kit	1 418 165
DIG Protein Labeling Kit	1 kit	1 367 200
Labeling nucleic acids		
Biotin Chem Link	1 set	1 812 149
Biotin High Prime	100 µl	1 585 649
Biotin RNA Labeling Mix	40 µl	1 685 597
DIG Chem-Link Labeling and Detection Set	1 set	1 836 463
DIG DNA Labeling Kit	1 kit	1 175 033
DIG Oligonucleotide 5'-End Labeling Set	1 set	1 480 863
DIG Oligonucleotide 3'-End Labeling Kit	1 kit	1 362 372
DIG High Prime	160 µl	1 585 606
PCR DIG Labeling Mix^{Plus}	2 × 250 µl	1 835 289
PCR ELISA DIG Labeling^{Plus}	2 × 250 µl	1 835 297
PCR DIG Probe Synthesis Kit	1 kit	1 636 090
DIG RNA Labeling Kit	1 kit	1 175 025

Additional related and required products

For Secondary detection- or Anti-species antibodies and Anti-DIG- or Anti-Fluorescein-conjugates please see **Roche Applied Science Biochemicals Catalog.**

4.3 Coating service

What the coating service offers

Customized Microplate Coating is a new service established to assist our customers. With this new service, customers can benefit from Roche's outstanding expertise in immunochemistry. Our proprietary coating technologies can be applied to diverse biomolecules of your choice:

- **Coating process according to GMP and ISO 9001**
- **Lot sizes of 200** (minimum) **to 2000 plates**
- **Very high production capacities**
- **Delivery time ≤ 8 weeks**
- **Broad range of solid support possible:**
  96-well and 384-well microplates (transparent, white, black)
  PCR plastic ware, plates or tubes
- **Common Coatings include:**
  Streptavidin, Anti-CAT, Anti-DIG, Anti-Fluorescein, Anti-ß-Gal, Anti-GST, Anti-HA (3 F 10), Anti-hGH, Anti-His6, Anti-Human IgG, Anti-Human-IgM, Anti-Mouse Ig, Anti-Rabbit IgG
- **Coating Procedure:**
  Various procedures available, depending on intended use: Nucleic acid or Protein screens. Samples will be provided.

Requirements and conditions for Customized coatings (e.g. antibodies, antigens or haptens) are available on request from our Coating Service department. Please contact our specialized local representative.

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On March 5, 1998, Boehringer Mannheim Biochemicals became Roche Molecular Biochemicals. Our new organization combines Boehringer Mannheim' s tradition of excellence in research reagents with Roche' s innovative expertise in molecular biology.

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EXHIBIT 8


EXHIBIT
5
PENGAD 800-631-6989

Anal. Chem. 1997, 69, 4939–4947

Accelerated Articles

Surface Plasmon Resonance Imaging Measurements of DNA Hybridization Adsorption and Streptavidin/DNA Multilayer Formation at Chemically Modified Gold Surfaces

Claire E. Jordan, Anthony G. Frutos, Andrew J. Thiel, and Robert M. Corn*

Department of Chemistry, University of Wisconsin–Madison, 1101 University Avenue, Madison, Wisconsin 53706

A combination of scanning and imaging surface plasmon resonance (SPR) experiments is used to characterize DNA hybridization adsorption at gold surfaces and the subsequent immobilization of streptavidin. Single-stranded oligonucleotides are immobilized at gold surfaces, and the hybridization of biotinylated complements from solution is monitored with SPR. The subsequent attachment of streptavidin to the biotinylated complements provides a method of enhancing the SPR imaging signal produced as a result of the hybridization and leads to a 4-fold improvement in the hybridization detection limit of the SPR imaging apparatus. In situ scanning SPR experiments are used to measure a 60 ± 20% hybridization efficiency between immobilized single-stranded DNA and biotinylated complements. From the information provided by both the in situ imaging and scanning SPR experiments, an absolute surface coverage of immobilized single-stranded DNA is estimated to be ~3×10^{12} molecules/cm^2. The SPR signal resulting from hybridization onto immobilized probes is further amplified by the formation of streptavidin/DNA multilayers which grow by a combination of DNA hybridization and biotin–streptavidin binding. DNA/DNA multilayers without streptavidin are used as an additional method of amplifying the SPR signal.

The detection of a specific DNA sequence from solution by hybridization adsorption onto an array of immobilized DNA probes has been studied extensively in recent years as a method of rapidly assaying mixtures of DNA sequences. Detection of hybridization onto a combinatorial mixture of oligonucleotides immobilized at surfaces has many potential applications including DNA sequencing, diagnosis of genetic diseases, and DNA computing.[1-6] All of these applications benefit from rapid methods of detecting and characterizing DNA hybridization adsorption with high specificity and signal-to-noise ratio. Surface plasmon resonance (SPR) is a promising technique for measuring hybridization of both labeled and unlabeled oligonucleotides in an in situ environment as the presence of DNA in solution does not interfere with the detection of hybridization at the surface. The commercially available Biacore instrument takes advantage of this characteristic of SPR and has been used by many researchers to investigate DNA hybridization in real time.[7-10] SPR can also be performed in an imaging geometry which allows it to simultaneously measure hybridization across an array of immobilized oligonucleotides.[10-12]

Many applications would benefit from the ability to detect small amounts of hybridization at surfaces, for example: polymerase

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S0003-2700(97)00976-1 CCC: $14.00

chain reaction (PCR) amplification typically provides nanomolar concentrations for detection, and some DNA computing applications have proposed hybridization to a single DNA sequence from a large combinatorial mixture immobilized on a surface.[6,13] Fluorescence is commonly used as a very sensitive technique for measuring small amounts of hybridized fluorescently labeled DNA; if evanescent wave fluorescence or scanning confocal fluorescent microscopy is used, it is even possible to detect hybridization adsorption in the presence of low concentrations of fluorescently tagged molecules in solution.[14-16] However, the amount of hybridization can be difficult to quantitate with fluorescence, particularly at gold surfaces where fluorescence quenching can occur.[17] In contrast, SPR is a technique that can be used to quantitatively measure the amount of hybridization that occurs in an in situ environment in real time.

SPR imaging is sensitive to small changes in the thickness or index of refraction of material at the interface between a thin gold film and a bulk solution.[18-21] SPR imaging has been shown to be a surface-selective technique that is sensitive *only* to molecules adsorbed to the interface.[22,23] This makes it possible to detect DNA hybridization adsorption in an in situ environment without the use of labels on the hybridized DNA, and SPR experiments of this type have been demonstrated elsewhere.[24] Although DNA hybridization can be measured by SPR without labeling, this method is sensitive only for surface coverages of immobilized single-stranded DNA (or probe DNA) of $\sim 10^{11}$ molecules/cm^2 or more. In order to improve the sensitivity of the SPR technique, a method of amplifying the SPR signal produced by DNA hybridization is developed here which involves binding streptavidin to biotin-labeled oligonucleotides hybridized to DNA immobilized at gold surfaces. This adsorption scheme is the reverse of the chemistry used in research reported previously which employs the binding between streptavidin and biotinylated oligonucleotides to immobilize DNA on streptavidin-coated surfaces with applications such as the purification of synthetic DNA, hybridization biosensors, and fabrication of MALDI mass spectroscopy samples.[25-30] Various methods of forming multilayers containing DNA have been reported previously,[31-34] and here we investigate two processes of DNA multilayer formation to further enhance the SPR sensitivity above that seen by the adsorption of a single layer of streptavidin.

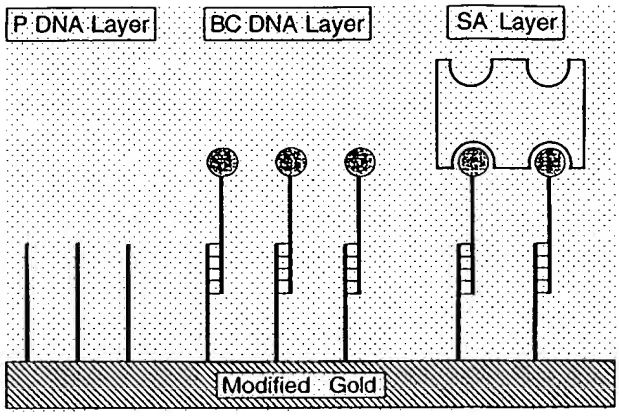

Figure 1. Schematic diagram showing DNA immobilization and hybridization, and streptavidin adsorption onto a modified gold surface: a layer of single-stranded probe DNA (P DNA), the probe DNA layer after hybridization of biotinylated complements (BC layer), and the biotinylated complement layer after adsorption of streptavidin (SA layer).

This paper describes the hybridization adsorption of biotinylated oligonucleotides to immobilized single-stranded DNA and the subsequent binding of streptavidin to these biotinylated complements. A thiol coupling surface chemistry that utilizes the self-assembly of alkanethiol monolayers[35-37] and has been presented in a series of previous papers was employed to immobilize single-stranded DNA at gold surfaces.[23,38-40] This resulted in a surface terminated with single-stranded DNA probes which are denoted as the "P DNA" layer in Figure 1. The DNA immobilization process has been characterized by a combination of polarization modulation-Fourier transform infrared reflection adsorption spectroscopy (PM-FTIRRAS) and scanning in situ SPR measurements. The probe DNA monolayer will hybridize to biotinylated complements (BC) from solution to form DNA duplexes terminated with biotin moieties as pictured in the "BC DNA" layer in Figure 1. Streptavidin can then be bound to the surface via the biotin moieties on the hybridized DNA, as depicted by the streptavidin (SA) layer in the figure. To aid in understanding how these layers form, in situ scanning SPR experiments have been used to characterize all three adsorbed biopolymers. SPR imaging experiments have also been used to investigate DNA hybridization and streptavidin adsorption onto probes with both perfectly

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matched and mismatched sequences. These SPR experiments indicate that the detection limit for DNA hybridization is significantly improved by the subsequent adsorption of streptavidin onto the biotinylated complements, and they have been used to estimate absolute surface coverages of the probe, biotinylated complements, and streptavidin. An additional set of SPR imaging experiments is used to demonstrate that the SPR signal resulting from the initial hybridization can be further increased by the formation of streptavidin/DNA multilayers. This multilayer deposition depends on a combination of both DNA hybridization and the streptavidin–biotin binding process. We also show in this paper that the SPR imaging signal can be enhanced by the formation of DNA/DNA multilayers that do not contain streptavidin.

EXPERIMENTAL SECTION

Materials. 11-Mercaptoundecanoic acid (MUA, Aldrich), poly(L-lysine)·HBr (PL, MW 14 000, Sigma), sulfosuccinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SSMCC, Pierce), streptavidin (Sigma), $NaHCO_3$ (Fluka), NaCl (Fluka), $NaHPO_4$ (Fluka), ethylenediaminetetraacetic acid (EDTA, Sigma), sodium dodecyl sulfate (SDS, Fluka), triethanolamine (TEA, Sigma), urea (Aldrich), and absolute ethanol (Pharmco) were all used as received. All oligonucleotides were synthesized by The University of Wisconsin Biotechnology Center and purified by reversed-phase binary gradient elution HPLC prior to use. Glen Research 5′-thiol-modifier C6 and 5′-biotin phosphoramidite were used in the synthesis of 5′-thiol- and 5′-biotin-modified oligonucleotides, respectively. Millipore-filtered water was used for all aqueous solutions and rinsing.

Surface Attachment Chemistry. The SPR experiments utilized thin (47 or 57 nm) gold films that had been vapor-deposited onto SF10 glass slides (18 × 18 mm^2, Schott Glass) as described previously.[41,42] The 47 and 57 nm gold films were used for in situ scanning and imaging experiments respectively. MUA monolayers were formed on gold films by immersing vapor-deposited gold surfaces into a 1 mM ethanolic solution for at least 18 h followed by thorough rinsing with ethanol and water. PL was adsorbed by immersing MUA-coated surfaces into a 0.7 mM PL solution in 5 mM $NaHCO_3$ for 30 min and then rinsing with water and ethanol. Thiol-terminated DNA was adsorbed to the PL surface by first exposing the entire surface to a 1 mM SSMCC solution in 0.1 M TEA, pH 7, for 15 min, then placing 0.6–1.8 μL drops of 0.5 mM thiol-terminated DNA in 0.1 M TEA, pH 7, on the surface, and allowing this to react overnight in a humid atmosphere. After reacting DNA onto the modified gold surfaces, slides were soaked in 300 mM NaCl, 20 mM $NaHPO_4$, 2 mM EDTA, pH adjusted to 7.8 (2×SSPE), and 0.2% SDS buffer for 1 h and thoroughly rinsed with water and ethanol before use for hybridization experiments. All oligonucleotide hybridization adsorption solutions contained 2 μM DNA in 2×SSPE, and streptavidin adsorption steps used 0.44 μM streptavidin in 2×SSPE unless otherwise noted. The modified gold films were thoroughly rinsed with either water or buffer after each adsorption step.

SPR Experiments. Both in situ scanning and imaging SPR experiments are described in this paper. An ex situ scanning SPR instrument has been described in detail previously,[19,40,43] and only small modifications to this instrument were required for in situ scanning SPR experiments. The ex situ scanning instrument was modified to perform in situ experiments by attaching a Kel-F flow cell with a 60 μL volume to the prism/sample assembly so that a 2 cm^2 area of the gold surface was in contact with solution. The other modification to the ex situ instrument involved replacing the BK7 prism with an SF10 prism ($n = 1.727$, Howard Johnson Optical Labs).[44] The in situ scanning SPR instrument generates plots (denoted as SPR curves) of the percent reflectivity (R) as a function of incident angle. The in situ imaging SPR experiments did not require any modifications to be made to the instrument described previously.[23,24] This instrument uses a CCD (iSight, iSC2050) camera to measure the R across a large incident beam and in this way generates an image of the surface. The percent reflectivity measured at a fixed incident angle can be related to the thickness of the material adsorbed to the surface so that different percent reflectivities will be measured from parts of the surface with different thicknesses.

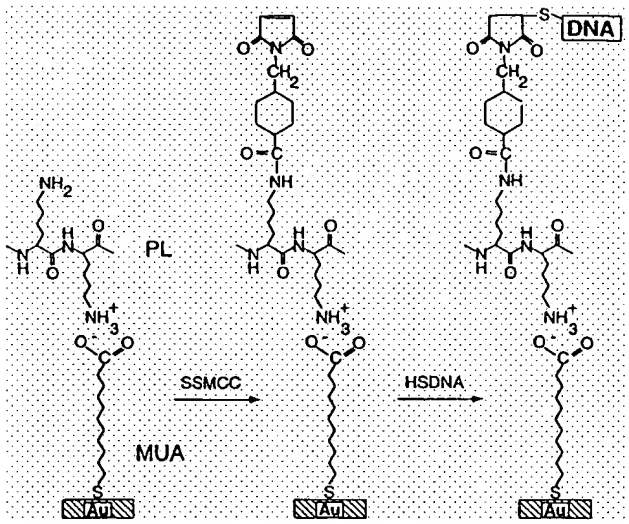

Figure 2. Surface modification scheme for attaching thiol-terminated DNA onto gold. Monolayers of 11-mercaptoundecanoic acid (MUA), and electrostatically bound poly(L-lysine) (PL) are deposited on a gold surface. The bifunctional linker SSMCC will react with some of the lysine residues on the PL to create a surface terminated with reactive maleimide groups, mal-PL surface. These maleimides will react with thiols, and in this way, it is possible to bind thiol-terminated DNA to the modified gold surface.

PM-FTIRRAS Measurements. PM-FTIRRAS spectra were obtained from a Mattson RS-1 spectrometer and a narrow-band HgCdTe detector using 3000 scans at 2 cm^{-1} resolution. The real-time interferogram sampling methods and optical layout have been described previously.[41,42,45] The PM-FTIRRAS differential reflectance values were converted to absorbance units for comparison with conventional IRRAS and FTIRRAS data.[43]

RESULTS AND DISCUSSION

A. Immobilization of Thiol-Terminated DNA onto Modified Gold Surfaces. Single-stranded DNA has been immobilized at vapor-deposited gold surfaces and the attachment chemistry was characterized with PM-FTIRRAS and SPR. The steps employed in the surface modification are shown in Figure 2. A gold

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surface was first coated with a self-assembled monolayer (SAM) of MUA and then exposed to a dilute PL solution, which resulted in the electrostatic adsorption of a monolayer of PL, as depicted in the leftmost structure in Figure 2.[40] This PL–MUA bilayer was used to minimize the nonspecific adsorption of DNA to the gold surface. The PL-modified surface was then exposed to a solution of the bifunctional linker SSMCC, which contains an *N*-hydroxysulfosuccinimide (NHSS) ester and a maleimide functionality. The NHSS ester end of the molecule reacted with some of the free lysine residues on the electrostatically adsorbed PL,[39] resulting in a PL surface containing reactive maleimide groups (mal-PL surface), depicted by the middle structure in Figure 2. PM-FTIRRAS experiments performed previously have estimated the percent of lysine residues on covalently bound PL that are modified by the SSMCC reaction. This estimate used the intensity of the 1708 cm^{-1} band, which is due to an in-phase stretch of the maleimide carbonyl groups.[39] Similar experiments (data not shown) performed on electrostatically adsorbed PL have shown that ~15% of the lysine residues were modified with maleimide groups. A surface coverage of 4×10^{14} lysine residues/cm^2 in an electrostatically adsorbed PL monolayer has been measured previously,[38] and from this number and the percent of modified lysine residues, a surface coverage of $\sim 6 \times 10^{13}$ maleimide groups/cm^2, is estimated. Maleimide functional groups react with thiols (sulfhydryl groups), and by this method, one is able to attach thiol-modified DNA oligonucleotides to the mal-PL surface, as shown by the final reaction in Figure 2.

The adsorption of thiol-modified DNA onto maleimide-functionalized surfaces has been followed by PM-FTIRRAS and in situ SPR. Figure 3 shows PM-FTIRRAS spectra for DNA immobilized on a gold surface and for bulk DNA. Figure 3a shows the difference between two PM-FTIRRAS spectra, one of a mal-PL surface and one of the same surface after the specific adsorption of thiol-terminated DNA. Figure 3b is a bulk spectrum of DNA obtained from a sample made by placing a DNA solution on a gold film and allowing the water to evaporate. The agreement between the positions and relative intensities of the bands in the two spectra indicate that thiol-terminated DNA can be immobilized at a maleimide-modified gold surface. The intensity of four IR absorption bands increases upon the immobilization of thiol-terminated DNA to a mal-PL surface. The assignments of the bands in Figure 3a are based on work reported in the literature:[46,47] the absorption at 1704 cm^{-1} is due to double-bond stretching vibrations of the DNA bases, the 1278 cm^{-1} band results from an NH bending vibration on the base thymine, and the bands at 1223 and 1073 cm^{-1} are respectively the antisymmetric and symmetric stretching vibrations of the phosphates. The small decrease at 1567 cm^{-1} is due to a shift in the amide II band of the mal-PL layer after the adsorption of DNA.

The sequences and symbols of oligonucleotides used in these experiments are shown in Table 1. The thiol-terminated probes that were immobilized at the gold surface are denoted as P1, P2, and P4. The 15 thymines adjacent to the thiol on the probes help to promote hybridization to complementary oligonucleotides by distancing the duplex-forming region from the surface.[2] The complements to these oligonucleotides are BC1, BC2, and C4a, respectively. The right-hand column in Table 1 contains cartoons

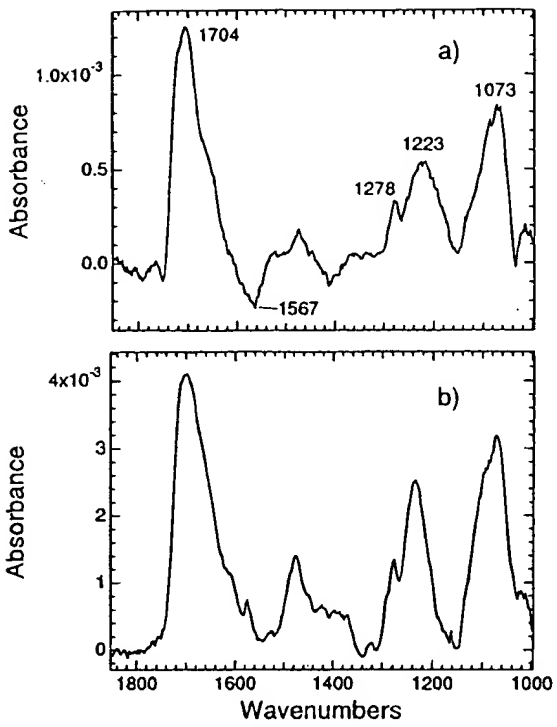

Figure 3. PM-FTIRRAS spectra of thiol-terminated DNA immobilized on a mal-PL surface and bulk DNA. (a) Spectrum showing the difference between two PM-FTIRRAS spectra, one of a mal-PL surface and one of the same surface after the specific adsorption of thiol-terminated DNA. (b) Bulk spectrum of DNA obtained from a sample made by placing a DNA solution on a gold film and allowing the water to evaporate. The similarity between the two spectra indicates that thiol-terminated DNA can be attached to a gold surface modified with maleimides.

indicating how hybridization occurs among these oligonucleotides. The modified gold surface is shown as a vertical line on the left of the cartoons, the horizontal lines represent DNA oligonucleotides, the shorter vertical lines indicate hybridization between perfectly matched complements, and the gray circles represent biotin moieties. Experiments using each of these oligonucleotides will be discussed in the following sections.

B. Scanning SPR Experiments on DNA Hybridization and Streptavidin Adsorption. Hybridization adsorption of biotinylated DNA onto oligonucleotides immobilized at gold surfaces and the subsequent binding of streptavidin have been investigated using in situ scanning SPR. Scanning SPR experiments performed in water were employed to characterize the formation of a mal-PL surface used to covalently attach thiol-terminated DNA. The shifts in the angle at which a minimum reflectivity is measured (SPR angle) as compared to that of bare gold are shown in Table 2. Also shown in the table are the total effective thickness and the additional increase in effective thickness after each adsorbed layer, along with indices of refraction for the adsorbed layers, bulk solutions, and the number of phases used in the Fresnel calculations (e.g., for $n = 5$ the phases were prism, gold, two phases of adsorbed film with different indices of refraction, and solution). Thicknesses calculated from SPR data are reported as effective thicknesses at a particular index of refraction since they measure an average thickness over the size of the incident beam and small errors in estimated indices of refraction can result in relatively large errors in absolute calculated thicknesses.[43,44] The effective thicknesses measured by in situ scanning SPR experi-

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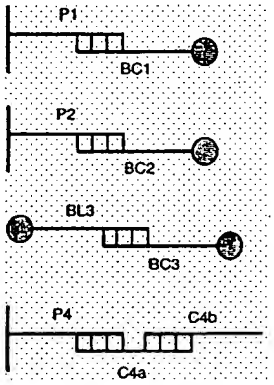
Table 1. Oligonucleotide Sequences and Hybridization Schemes

symbol	sequence	hybridization cartoon[a]
P1	5' thiol $(T)_{15}$ GCT TTC CTG AAG TTC G 3'	
BC1	5' biotin CGA ACT TCA GGA AAG C 3'	
P2	5' thiol $(T)_{15}$ GCT TCA ACA CCA TTC G 3'	
BC2	5' biotin CGA ATG GTG TTG AAG C 3'	
BL3	5' biotin $(T)_5$ CGA ACG AAG TTG GTT GAA GC 3'	
BC3	5' biotin $(T)_5$ GCT TCA ACC AAC TTC GTT CG 3'	
P4	5' thiol $(T)_{15}$ CGA ACG AAG TTG GTT GAA GC 3'	
C4a	5' GCT TGC TTG AAG GAA GTT CGG CTT CAA CCA ACT TCG TTC G 3'	
C4b	5' CGA ACT TCC TTC AAG CAA GCC GAA CGA AGT TGG TTG AAG C 3'	

[a] Oligonucleotides on top of each cartoon are shown left to right 5′ to 3′ and those on the bottom are shown 3′ to 5′.

Table 2. SPR Angle Shifts and Calculated Film Thicknesses

layer	total $\Delta\theta$ (deg)[a]	solution n[b]	layer n	no. of phases[c]	effective total thickness (Å)	effective additional thickness (Å)[d]
MUA	0.147 ± 0.018[e]	1.333	1.40[f]	4	17.0 ± 1.4[e]	17.0
PL	0.279	1.333	1.52[f]	5	27.0	10.0
SSMCC	0.294	1.333	1.52[f]	5	28.3	1.3
P2	0.457	1.336	1.46[g]	6	45.8	17.5
BC2	0.503	1.336	1.46[g]	6	51.0	5.2
SA1	0.715	1.336	1.45[f]	7	75.2	24.2

[a] Total shift in SPR angle from that of bare Au. [b] Measured by refractometry. [c] Number of phases used in Fresnell calculation when effective thicknesses are determined. [d] Increase in the effective thickness from the previous layer. [e] Total $\Delta\theta$ and thickness values are the average of three samples with the errors resulting from sample-to-sample variation; they do not include errors from estimating n. [f] Index of refraction estimated from bulk values. [g] From ref 11.

ments for MUA and PL layers are 17.0 and 10.0 Å, respectively; these values compare reasonably well with previous ex situ measurements on similar surfaces.[40] Upon the adsorption of SSMCC to the PL surface, a change in thickness of only 1.3 Å was observed. This small increase is reasonable for a surface coverage of 6×10^{13} maleimide groups/cm^2 as estimated from the PM-FTIRRAS data.

Figure 4 shows the experimental SPR curve for a mal-PL surface on which the thiol-terminated oligonucleotide P2 has been adsorbed (open circles) and the solid line is a six-phase Fresnel fit to the data. This and all subsequent in situ experiments were performed in 2×SSPE, which promoted DNA hybridization. The inset in Figure 4 is an expanded view of the SPR minima for the P2 surface and the same surface after the hybridization adsorption of perfectly matched biotinylated complements (BC2, open squares). The calculated effective thicknesses of these two layers are shown in Table 2. P2 has 31 nucleotide bases (31-mer), and the thickness of the P2 layer measured in 2×SSPE is 17.5 Å. As the surface coverage of maleimides is only ~6×10^{13} molecules/cm^2, it is expected that the surface attachment chemistry would form only a partial monolayer of probe DNA oligonucleotides. The measured thickness of the P2 layer is in agreement with a partial monolayer as a fully packed layer of an extended 31-mer oligonucleotide is estimated to be ~100 Å thick by assuming the single-stranded DNA will have the same length per base as double-stranded DNA. The hybridization of a layer of biotinylated complements, BC2, shows an additional increase in the effective thickness of 5.2 Å. This is ~30% of the effective thickness measured for P2 in 2×SSPE, and since BC2 is about half the size of P2, a hybridization efficiency of 60 ± 20% is determined. This hybridization efficiency was not found to be highly sequence dependent as similar shifts in SPR angles were observed for the adsorption of P1 and BC1. A hybridization efficiency of ~60% is in good agreement with those measured by other researchers on similar systems, who have found hybridization efficiencies between 40 and 80% for DNA oligonucleotides immobilized to gold, silicon, and carboxy-methylated dextran.[9,10,48,49]

In situ scanning SPR experiments have also shown that it is possible to specifically adsorb the protein streptavidin onto biotinylated DNA hybridized onto immobilized oligonucleotide probes, as depicted by the rightmost cartoon in Figure 1. The open triangles in Figure 4 are the SPR curve taken after streptavidin has been adsorbed onto BC2. The expanded view of the SPR minima shown in the inset to Figure 4 clearly indicates that the SPR angle has shifted significantly upon the adsorption of streptavidin as compared to the smaller shift seen for just the biotinylated complements. The calculated effective thickness increase for the streptavidin adsorption is 24.2 Å, and this is a 5-fold increase in the effective thickness of streptavidin and BC2 as compared to just BC2. A 24.2 Å thick streptavidin film

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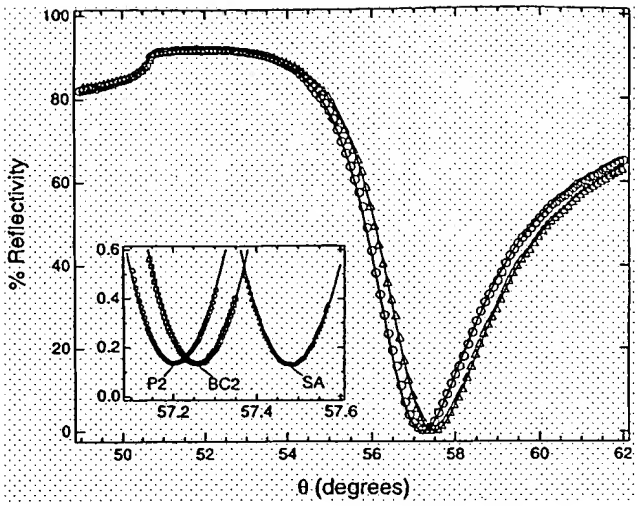

Figure 4. In situ scanning SPR curves of DNA immobilization and hybridization and streptavidin adsorption, taken in 2×SSPE buffer. The full SPR curves are shown after the deposition of the single-stranded probes and streptavidin. The inset is an expanded view of the SPR minima for the probe (P2), hybridized biotinylated complement (BC2), and streptavidin (SA) layers. The open circles, squares, and triangles show respectively the experimental percent reflectivities for P2, BC2, and SA as a function of incident angle, θ. The solid lines are the results of six- or seven- (see Table 2) phase complex Fresnel calculations for each layer, and the shift in the angle of the minimum R is due to differences in thickness and index of refraction between the layers.

corresponds to about half of a monolayer based on the size of streptavidin (54 Å × 58 Å × 48 Å).[50,51] Because avidin and streptavidin have similar dimensions, a surface coverage of 1.5×10^{12} molecules/cm^2 for half a monolayer of streptavidin is estimated based upon the previously measured surface coverage for a full monolayer of avidin of 3×10^{12} molecules/cm^2.[38] The additional thickness of the adsorbed streptavidin is probably useful for scanning SPR measurements on very small amounts of hybridized DNA, but the enhancement is even more important for fixed-angle SPR imaging experiments.

C. Imaging SPR Experiments on DNA Hybridization and Streptavidin Adsorption. Imaging SPR experiments are performed at a fixed angle and can simultaneously measure the percent reflectivity (R) across an entire surface patterned with areas of differing thickness. Since imaging SPR experiments are performed at a single fixed angle, they are less sensitive than scanning SPR experiments, which use an average over many angles to determine an effective thickness. For this reason they benefit significantly from amplifying the SPR response by streptavidin adsorption. Figure 5a shows the arrangement of P1 and P2 spots adsorbed to a mal-PL surface (mal-PL/probe surface) used in the following experiment. The sequences of P1 and P2 differ by 8 out of 16 bases; therefore, the complement to each will be an 8-base mismatch with the other. Figure 5b is an image of a mal-PL/probe surface to which BC1 and streptavidin have been adsorbed (taken in 2×SSPE buffer), and Figure 5c shows a surface to which BC2 and streptavidin are bound. Because the images in Figure 5 were taken at an angle below the SPR angle, a higher R is expected for the thicker layers, as represented by the red areas. From Figure 5, it is possible to qualitatively see that adsorption of biotinylated complements and streptavidin significantly increases the SPR response in the areas containing the corresponding perfectly matched probes. SPR images can be represented quantitatively by vertically averaging the R values measured at each pixel of the CCD camera within a rectangle which crosses the center of two of the spots, thus generating a "line profile" across the spots.[23] For example, the dashed lines in panels a and b of Figure 6 are line profiles generated through the top two spots of the images in panels b and c of Figure 5, respectively.

By quantitatively analyzing the SPR imaging data for a series of images showing biotinylated DNA hybridization and streptavidin binding, it is possible to measure the increase in sensitivity due to streptavidin adsorption. Figure 6 shows line profiles generated from such a series of images, taken in 2×SSPE. A line profile generated from an image of the initial mal-PL/probe surface is shown as the solid line in Figure 6a. The R measured for the mal-PL background and the probes are in agreement with the R expected from scanning in situ measurements and the angle at which the images were taken. The dot–dash line in Figure 6a is a line profile taken after this surface was exposed to a 2 μM BC1 solution and shows a ~2.5% increase in the R measured from the area containing P1; essentially no change is seen in the P2 or the mal-PL areas. This indicates that BC1 is hybridizing with P1 instead of nonspecifically adsorbing to the surface. Upon exposure of the mal-PL/probe/BC1 surface to a 0.44 μM streptavidin solution (dashed line), a relatively large increase of 10% R above the probe surface in the area containing BC1 is observed with no change in R measured elsewhere. It is seen (data not shown) that the line profile returns to the R measured for the initial probes (solid line Figure 6a), if this surface is exposed to 8 M urea at 31 °C. This indicates that all of the BC1 and streptavidin layers are removed, leaving only the DNA probes on the surface. The fact that BC1 and streptavidin are completely removed by urea, which disrupts the hydrogen bonding involved in the hybridization,[52] is further evidence that it is hybridization by which BC1 is adsorbed and that the streptavidin binds specifically to the biotin on the BC1. This will allow the surface containing the DNA probes to be used for multiple hybridization experiments, which is a useful feature for DNA adsorption biosensors. After the surface was regenerated with urea, the sample was exposed to BC2 and then to streptavidin. Figure 6b shows the stepwise hybridization of BC2 onto the P2 area (dot–dash line in Figure 6b) and the subsequent binding of streptavidin to BC2 (dashed line). The adsorption of streptavidin to hybridized biotinylated DNA increases the R measured as a result of the hybridization by a factor of 4. This increase in the R is slightly less than the thickness increase measured by in situ scanning SPR because the R measured at this angle is nonlinear with thickness. However, the enhancement still increases the sensitivity of the SPR imaging experiment enough that hybridization of BC1 and BC2 onto only the perfectly matched probes is easily observed, as shown in Figure 6.

Absolute surface coverages of the immobilized probe DNA can be estimated from the scanning experiments presented in section B and an additional in situ imaging experiment (data not shown). In this additional experiment, 2 μM biotinylated DNA and 2 μM

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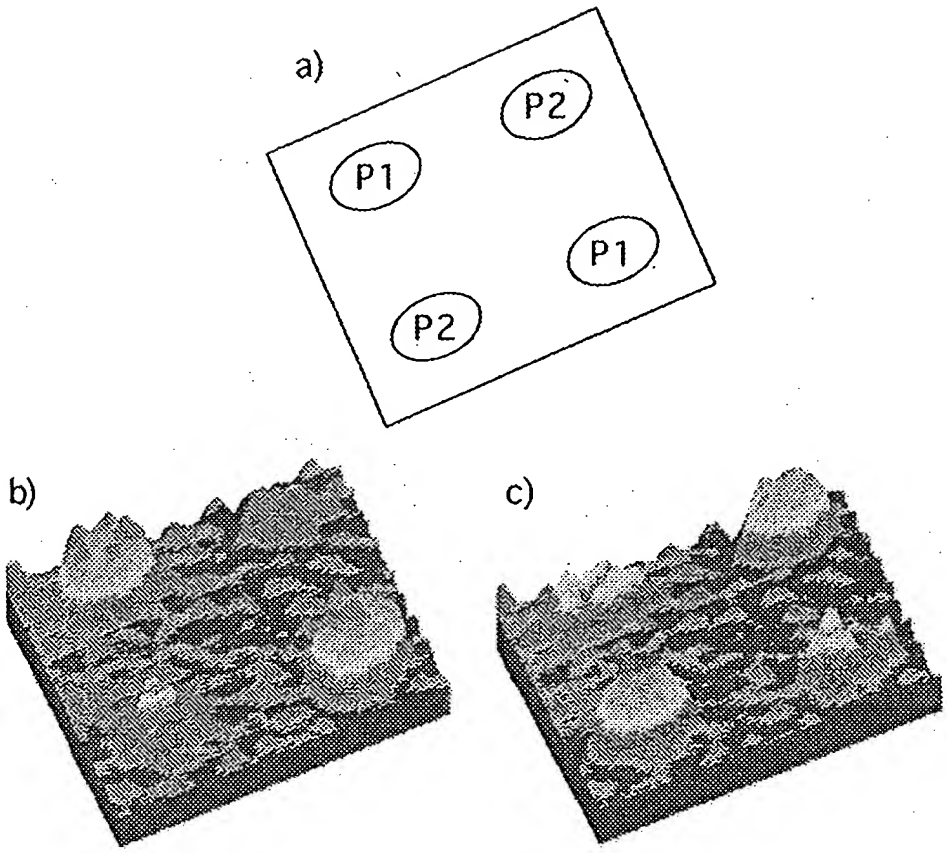

Figure 5. In situ SPR images of hybridization and streptavidin adsorption onto two different probe oligonucleotides, P1 and P2. (a) Diagram showing the placement of the probe spots on a mal-PL surface. (b) SPR image taken after hybridizing BC1 onto P1 and adsorbing streptavidin. (c) SPR image of the same surface after regenerating it with urea then hybridizing BC2 onto P2 and adsorbing streptavidin.

streptavidin were mixed in solution and then adsorbed by hybridization onto oligonucleotides immobilized on a gold surface. The identical R was measured by this method and by sequentially exposing the same surface to first biotinylated DNA and then streptavidin. This indicates that the same amount of streptavidin is adsorbed to the surface by either method. Because the interaction between biotin and streptavidin is so strong ($K_{binding} = 10^{15}$),[51] when stoichiometric amounts of streptavidin and biotinylated DNA are mixed in solution and then adsorbed to immobilized probes, all of the biotinylated DNA will be bound to streptavidin. Since either method will adsorb only half of a monolayer of streptavidin, it must also be true that all of the biotinylated complements interact with streptavidin when a sequential method of adsorption is employed.

From the fact that all of the biotinylated oligonucleotides bind to streptavidin and information provided by the in situ scanning experiments, it is possible to make an order of magnitude estimate of the absolute surface coverage of thiol-terminated probes adsorbed to a mal-PL surface. In situ scanning experiments have determined the absolute surface coverage of streptavidin to be $\sim 1.5 \times 10^{12}$ molecules/cm^2, and the hybridization efficiency between the probes and the biotinylated complements to be 60 ± 20%. From the surface coverage of streptavidin and the fact that all of the biotinylated complements are bound to one of streptavidin's four binding sites, a surface coverage for the biotinylated complements is estimated to be 1.5×10^{12} molecules/cm^2. Then using the hybridization efficiency, the surface coverage of immobilized DNA is estimated to be 3×10^{12} molecules/cm^2. This is in good agreement with surface coverages measured for other methods of immobilizing DNA, which generally range from 3.0×10^{12} to 1.5×10^{13} molecules/cm^2.[10,48,53]

The adsorption of streptavidin onto biotinylated DNA duplexes immobilized at a gold surface as discussed in this section will increase the SPR signal produced as a result of the hybridization. This application of biotinylated DNA and streptavidin binding is the reverse of what most researchers have done previously. Most other applications have used surfaces to which streptavidin is bound to initially immobilize biotinylated oligonucleotides.[25-30] The fact that these researchers have used streptavidin to attach DNA to surfaces suggests that it may be possible to bind a second layer of biotinylated DNA to the streptavidin layers discussed in this paper. The following section describes experiments investigating this as a possible method of forming multiple layers of streptavidin and DNA.

D. Formation of DNA Multilayers. (a) Streptavidin/DNA Multilayers. Although one step of streptavidin adsorption causes a significant increase in the SPR signal observed due to the hybridization of biotinylated DNA, it is possible to further amplify this signal by the formation of streptavidin/DNA multilayers. The streptavidin/DNA multilayers are formed by first making a streptavidin-coated surface as discussed above and then sequentially adsorbing two strands of complementary biotinylated linker oligonucleotides (BL3 and BC3 shown in Table 1) followed by a second layer of streptavidin as shown in the inset of Figure 7. Figure 7 shows the differential R measured during the formation of streptavidin/DNA multilayers on a spot of P2 deposited on a

(53) Ito, K.; Hashimoto, K.; Ishimori, Y. *Anal. Chim. Acta* **1996**, *327*, 29–35.

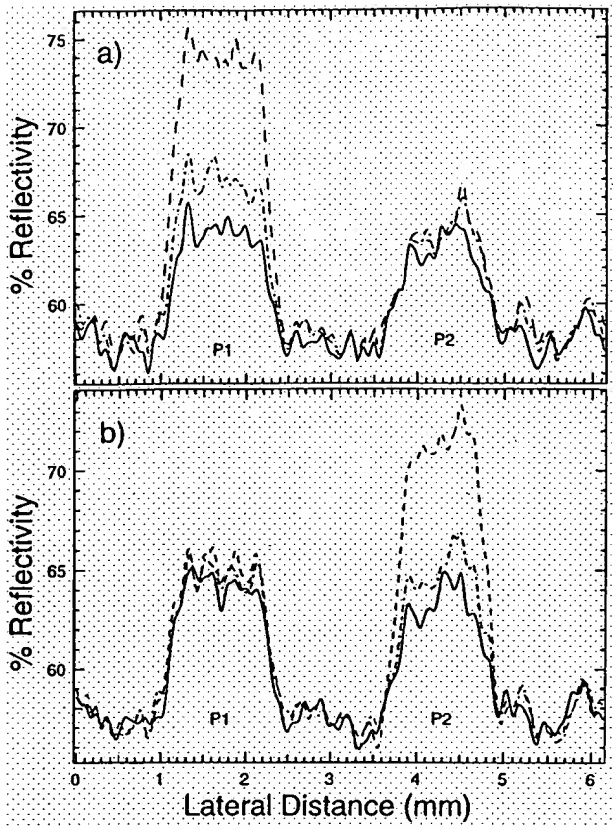

Figure 6. Line profiles showing hybridization and streptavidin adsorption onto areas of P1 and P2 immobilized at a mal-PL surface. (a) The solid line is the R measured from a mal-PL surface to which the two DNA probes have been attached. The dot–dash line is from the same surface after exposing it to a solution of BC1 and shows a small increase in R only in the P1 area. The dashed line is a line profile taken after exposing the surface to streptavidin and shows a relatively large increase in R in the P1 area. (b) The solid line is the R measured from the same surface shown in (a) after removing all of the BC1 and streptavidin with urea. The dot–dash and dashed lines in (b) are respectively the R measured after exposing the surface to BC2 and streptavidin. The comparatively large increase in R observed after exposing a surface having hybridized biotinylated complements on it to streptavidin shows that the adsorption can be used to significantly amplify the SPR imaging signal produced by the hybridization.

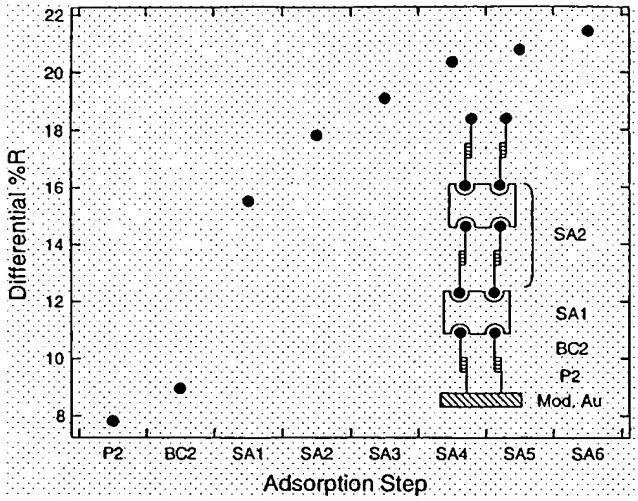

Figure 7. The differential percent reflectivity measured between a spot of DNA probe, P2, and a mal-PL surface as streptavidin/DNA multilayers are built up on the P2 spot. The inset is a schematic diagram showing how the streptavidin/DNA multilayers are deposited. After the first streptavidin adsorption step the increase in R for the biotinylated DNA binding and hybridization is so small that only the total differential R after depositing two biotinylated oligonucleotides and streptavidin is shown for the adsorption steps, SA2–SA6. This provides a method for further increasing the SPR imaging signal above that observed for a single streptavidin layer.

mal-PL surface. The differential reflectivity for these experiments is measured as the difference in the R for the mal-PL background and the P2 spot observed after each adsorption step. The additional R measured for the adsorption of BL3 and BC3 is very small and so only the total increase in R after the adsorption of BL3, BC3, and streptavidin is shown as the streptavidin adsorption step (SA2), in Figure 7. These three adsorption steps can be repeated to form multiple streptavidin/DNA layers as has been demonstrated for six streptavidin adsorption steps (SA1–SA6). It has also been shown that no measurable adsorption occurs to the mismatch P1 and that the streptavidin/DNA multilayers can be completely removed from the surface by exposing it to 8 M urea at 31 °C (data not shown). Several factors indicate that multilayer formation occurs by a combination of DNA hybridization and biotin–streptavidin binding. These include the ability to remove the multilayers with urea, the observation that no nonspecific adsorption occurs on the mismatch, P1, or the mal-PL background, and the fact that multilayer formation does not occur unless the streptavidin surface is exposed to both BL3 and BC3 before depositing a second layer of streptavidin. The ability to form streptavidin/DNA multilayers using this scheme indicates that at least some of the biotin sites on the adsorbed streptavidin are active, which is an important point as adsorbing biological molecules to surfaces sometimes changes their activity.[54]

Figure 7 shows that the increase in R measured after six streptavidin adsorption steps is about twice as much as that measured for a single streptavidin amplification. This indicates that the SPR imaging signal can be further increased by streptavidin/DNA multilayer formation; however, the change in R becomes smaller for each subsequent streptavidin layer. Part of this smaller change in R is due to the nonlinear relationship between R and thickness at the angle the experiment was performed. However, this will not account for all of the change in R, indicating that there is a real decrease in additional thickness between subsequent streptavidin adsorption steps. Hybridization efficiencies between complementary DNA oligonucleotides of less than 100% and the possibility of some biotin binding sites being inactive are both expected to contribute to the deposition of less streptavidin with each adsorption step. Despite this decrease in additional thickness, it is possible to form at least six streptavidin/DNA multilayers which further improves the SPR imaging signal that can be produced from the initial hybridization of biotinylated DNA.

The combination of DNA hybridization and streptavidin–biotin binding has been used previously to increase the amount of signal observed from hybridization adsorption or to form macroscopic structures. Caruso et al. have shown that multilayer formation will occur via the successive deposition of avidin and poly(stryenesulfonate) and that once these multilayers are formed it is possible to bind a biotin-labeled oligonucleotide to the avidin

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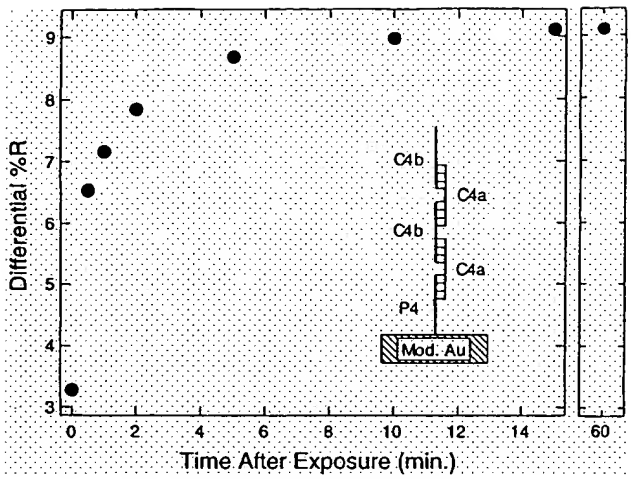

Figure 8. Differential percent reflectivity between a spot of DNA probe, P4, and a mal-PL surface measured as a function of time after exposing the surface to a solution containing a mixture of the oligonucleotides C4a and C4b. The inset is a schematic diagram showing the formation of DNA/DNA multilayers. The differential R increases rapidly for the first 5 min and then slowly levels out until it reaches a constant value after ~10 min. DNA/DNA multilayer formation is expected to depend on the efficiency and kinetics of the hybridization and may provide a novel method for investigating these quantities by SPR imaging.

in these multilayers and subsequently hybridize another oligonucleotide to this immobilized DNA.[32] DNA hybridization has also been used to reversibly form macroscopic aggregates of colloidal gold. This has been done by coating the particles with two different thiol-terminated oligonucleotides, then, if an oligonucleotide duplex is added that has one end complementary to each of the immobilized DNA sequences, the particles will aggregate.[55] These colloidal gold particles aggregate in a single solution, suggesting that perhaps streptavidin/DNA multilayers at gold surfaces could also be formed from a single adsorption solution. However, attempts to form streptavidin/DNA multilayers from a solution containing a mixture of BL3, BC3, and streptavidin were unsuccessful. For this reason, an alternative method of forming multilayers from a single adsorption solution has been developed.

(b) DNA/DNA Multilayers. The formation of DNA/DNA multilayers without streptavidin present has been investigated as a means of amplifying the SPR signal produced by hybridization using a single adsorption solution. This scheme involves assembling multilayers of two DNA oligonucleotides (C4a and C4b), as shown in the inset of Figure 8. The hybridization occurs as shown in Table 1, where half of C4a at the 5′ end is complementary to half of C4b at the 3′ end and the other half of C4a is complementary to the 5′ end of C4b. In preliminary experiments, a spot of the thiol-terminated oligonucleotide P4, which is complementary to half of C4a at the 3′ end, was attached to a mal-PL surface as described in section A. This surface was then exposed to a 2×SSPE solution having a 4 μM concentration of each C4a and C4b. Figure 8 shows the differential R between the P4 spot and the mal-PL background measured as a function of time after exposing the surface to the C4a and C4b solution. From the figure it can be seen that the differential R increases quickly for the first 5 min and then after ~10 min levels off to a constant R which is ~9% higher than the mal-PL background. This increase is due to the adsorption of multiple strands of C4a and C4b onto each P4 oligonucleotide. The imaging experiments shown in Figure 8 were performed at a fixed angle different from that for the experiments shown in Figures 5–7, so no direct comparisons between the increase in R produced by streptavidin/DNA and DNA/DNA multilayers can be made. Although the amount of adsorption due to DNA/DNA multilayer formation has not been quantified, it can be seen that this provides a means of increasing the R due to hybridization using a single amplification solution. It is also expected that the rate at which this multilayer formation occurs is related to the hybridization rate for C4a and C4b and that this could provide a novel method of measuring DNA hybridization kinetics with in situ SPR imaging. Further investigation of the formation of DNA/DNA multilayers and their possible applications to the study of hybridization efficiency and kinetics are left for future experiments.

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CONCLUSIONS

Hybridization adsorption of biotinylated oligonucleotides on gold surfaces has been characterized by in situ scanning and imaging SPR, and the subsequent adsorption of streptavidin as a method of amplifying the SPR signal produced by the hybridization has been investigated. In situ scanning SPR experiments have measured a hybridization efficiency of 60 ± 20% for immobilized oligonucleotide probes and perfectly matched biotinylated complements. The adsorption of streptavidin has been shown to occur specifically to biotinylated DNA, and both streptavidin and biotinylated DNA can be removed by disrupting the hybridization. The specific adsorption of streptavidin to biotinylated DNA allows streptavidin adsorption to be used as a method of amplifying the SPR imaging signal produced by the hybridization of biotinylated DNA to oligonucleotides immobilized at gold surfaces. This amplification improves the detection limit for DNA hybridization using SPR imaging by a factor of ~4. Using a combination of the in situ scanning and imaging experiments, it is possible to estimate surface coverages for the thiol-terminated probe, biotinylated complement, and streptavidin.

The formation of hybridization dependent multilayers has also been investigated. It has been shown that streptavidin/DNA multilayers, up to at least six streptavidin layers, can be formed and that these multilayers grow by a combination of both DNA hybridization and streptavidin–biotin binding. This multilayer formation can be used to further amplify the signal produced by the initial hybridization above that observed from a single streptavidin layer. DNA/DNA multilayers without streptavidin have also been deposited. Future experiments will investigate the formation of these multilayers as a novel method for measuring DNA hybridization efficiency and kinetics by SPR imaging.

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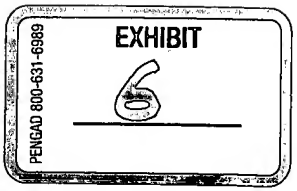
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Real-time detection of DNA hybridization and melting on oligonucleotide arrays by using optical wave guides

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Contributed by John D. Baldeschwieler, February 3, 1995

ABSTRACT **The challenge of the Human Genome Project is to increase the rate of DNA sequence acquisition by two orders of magnitude to complete sequencing of the human genome by the year 2000. The present work describes a rapid detection method using a two-dimensional optical wave guide that allows measurement of real-time binding or melting of a light-scattering label on a DNA array. A particulate label on the target DNA acts as a light-scattering source when illuminated by the evanescent wave of the wave guide and only the label bound to the surface generates a signal. Imaging/visual examination of the scattered light permits interrogation of the entire array simultaneously. Hybridization specificity is equivalent to that obtained with a conventional system using autoradiography. Wave guide melting curves are consistent with those obtained in the liquid phase and single-base discrimination is facile. Dilution experiments showed an apparent lower limit of detection at 0.4 nM oligonucleotide. This performance is comparable to the best currently known fluorescence-based systems. In addition, wave guide detection allows manipulation of hybridization stringency during detection and thereby reduces DNA chip complexity. It is anticipated that this methodology will provide a powerful tool for diagnostic applications that require rapid cost-effective detection of variations from known sequences.**

Sequencing by hybridization (SBH) is a revolutionary technique for the generation of nucleic acid sequence information (1–6). A single hybridization experiment allows examination of a large number of different sites on a DNA molecule. Diagnosis of several human genetic conditions such as Duchenne muscular dystrophy (7, 8) or cystic fibrosis (9) will require the resolving power of an SBH type system to determine the mutation associated with the disease state in a cost-effective manner. One case in point is cystic fibrosis where >300 mutations have been identified (10).

SBH uses a large number of oligonucleotides immobilized in a high-density two-dimensional array and is particularly suited to multiplex applications. Such a device has been called a "DNA chip" analogous to the high-density circuits produced by the electronics industry (11, 12). A sample of unknown DNA is applied to the chip and the pattern of hybridization is determined and analyzed to obtain sequence information (13, 14).

Most of the DNA hybridization detection methods employed thus far use radioactive (15–20), enzyme-based chemiluminescent (21), or fluorescent (22) labels. Detection and measurement can be accomplished with phosphor systems (23) or, for the latter two labels, with charge-coupled device (CCD) cameras (24), cooled CCD cameras, image intensifiers coupled to CCD cameras, or a photomultiplier tube coupled with mechanical raster scanning (12, 25). In a typical SBH experiment, a labeled DNA sample is applied to the chip to allow hybridization. Excess label/DNA may be washed from the chip surface to minimize background. By use of a confocal microscope, the measurement of the fluorescent light is confined to the surface of the chip and the washing step is not required (26). Because the amount of fluorescent label on the surface of a chip is quite low, the time required to scan the array is on the order of 1 min. Such integration times are also typical for cooled CCD camera systems. Much higher DNA densities can be achieved by using a gel matrix and in this case the fluorescent signal can be read at standard speed with a low-sensitivity CCD camera (24). However, the gel system affects the kinetics of hybridization/melting through multiple binding events in the three-dimensional matrix of immobilized DNA and requires a washing step (27).

Melting curves could provide an additional dimension to the system and allow differentiation of closely related sequences, a concern in implementation of SBH technology (28). The ability to change temperature and monitor the chip hybridization patterns would also be useful in cases where there is a wide variation in GC content and may obviate the need for agents like tetramethylammonium chloride (17). However, if 1 min is required to read/wash a DNA chip, then a high-resolution melting curve from 30 to 70°C would require 40 min; i.e., measurement is rate limiting. Removal of background signal would require some sort of washing system to eliminate the label as it dissociates from the capture site.

The present report suggests the use of a two-dimensional optical wave guide and light scattering labels to detect hybridization patterns. While generation of scattering signals using an optical wave guide is not new (29), to our knowledge, the use of a wave guide with an array of binding sites has not been reported. The evanescent wave created by the wave guide is used to scatter light from a particulate label adsorbed at multiple DNA capture zones placed on the wave guide surface. Since an evanescent wave only extends a few hundred nanometers from the wave guide surface (30), the unbound/dissociated label does not scatter light and a wash step is not required. The signal intensity is sufficient to allow measurement of the surface binding and desorption of the light scattering label can be studied in real time; i.e., detection is not rate limiting. The hybridization pattern on the chip can be evaluated visually or acquired for quantitative analysis by using a standard CCD camera with an 8-bit video frame grabber in 1/30 of a second.

MATERIALS AND METHODS

Chips. DNA chips for radioactive detection were prepared by washing glass microscope slides with Ivory soap (Procter & Gamble), rinsing with water, heating at 80°C with concentrated sulfuric or nitric acid for at least 30 min, rinsing, and storing

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Abbreviations: SBH, sequencing by hybridization; CCD, charge-coupled device.

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in deionized water. Slides were then treated with 1% glycidoxypropyl silane (Aldrich) in 95% ethanol (pH adjusted to 5.5 with sulfuric acid) for 1 h. Excess silane was removed by dipping the slide in 95% ethanol for 1 min, and the slide was dried by baking at 150°C for 20 min. Silane-treated slides were cooled and stored in an argon-filled desiccator until use. The 3′-amino linked oligonucleotides (Table 1) from Synthecell were diluted to 50 μg/ml in PBS (10 mM sodium phosphate, pH 7.4/120 mM NaCl/2.7 mM KCl; Sigma), placed as 1-μl drops on the slides, and allowed to dry. After at least 1 h, the slides were rinsed and stored in TE (TE = 10 mM Tris·HCl, pH 7.5/0.5 mM EDTA). DNA density was 30–300 molecules per μm^2 as determined by autoradiography with oligonucleotides of known specific radioactivity.

DNA chips for wave guide detection were constructed by using presynthesized 3′-amine-labeled oligonucleotides obtained from Synthecell or Genosys. Glass substrates (no. 2 microscope cover slides, Corning, or equivalent) were cleaned by soaking in 2 M NaOH for 1 h followed by rinsing with HPLC grade water (Fisher). The glass was protein-coated by application of 0.05% casein (Abbott) for 1 min. The casein solution was flushed from the surface by using a wash bottle. The 3′-amine oligonucleotides were rehydrated with 50 μl of water (Table 1) and then diluted 1:20 into PBS for spotting onto the casein-coated slides. The oligonucleotide solutions were applied to the slide with a pipette in a 0.5-μl spot or by dipping the flat end of a drill blank "pin" (HSS 67, Hayden Twist Drill, Warren, MI) into the DNA solution and touching it to the slide surface. The latter procedure was automated by using an X-Y-Z table (Asymtek). After drying, the excess DNA was washed from the chip. In some cases a second glass slide was fixed to the wave guide to form a channel that holds the sample or conjugate solutions (Fig. 1*A*).

Selenium Colloid and Conjugate. Selenium colloid was produced by addition of 4 ml of 1% sodium ascorbate (Sigma) to 200 ml of boiling water followed by addition and rapid mixing of 2 ml of 1% selenium dioxide (Aldrich) (32). The colloid used in these studies had a particle size of ≈0.2 μm, a pH of 5.4, an OD_{546} of 32, and an absorption maximum at 546 nm. Selenium-antibody conjugate was prepared fresh before use by mixing of 2.5 μl of anti-biotin (polyclonal rabbit anti-biotin, 1.13 mg/ml in PBS) to 1 ml of selenium colloid followed by addition of 30 μl of 20% (wt/vol) bovine serum albumin (Sigma).

Hybridization and Staining for Wave Guide. Solutions of 3′-biotinylated DNA (Table 1) were diluted 1:1000 into 1% casein/10 mM Tris·HCl, pH 7.8/15.4 mM NaCl and applied to the surface of the chip for 5 min at room temperature (23–25°C). The DNA solution was drained off and, without allowing the surface to dry, a solution of anti-biotin selenium

Table 1. DNA sequences for chip construction and hybridization experiments

DNA no.	Sequence	Concentration, μM
	Immobilized on the chip	
1	5′-TATCATCTTTGGTGT-3′-NH_2 (ΔF508WT)	139
2	5′-AATATCATTGGTGTT-3′-NH_2 (ΔF508)	169
3	5′-AGTGGAGGTCAACGA-3′-NH_2 (G551D WT)	154
4	5′-AGTGGAGATCAACGA-3′-NH_2 (G551D)	282
5	5′-AGGTCAACGAGCAAG-3′-NH_2 (R553X WT)	127
6	5′-AGGTCAATGAGCAAG-3′-NH_2 (R553X)	139
7	5′-TGGAGATCAATGAGC-3′-NH_2 (G551D + R553X)	31
8	5′-TGGAGATCAACGAGC-3′-NH_2 (G551D + R553X WT)	18
9	5′-TGGAGGTCAATGAGC-3′-NH_2 (G551D WT + R553X)	74
	Complementary sequences for radioactive detection	
11	5′-ACACCAAAGATGATA-3′	112
12	5′-AACACCAATGATATT-3′	119
13	5′-TCGTTGACCTCCACT-3′	219
14	5′-TCGTTGATCTCCACT-3′	214
15	5′-CTTGCTCGTTGACCT-3′	226
16	5′-CTTGCTCATTGACCT-3′	200
17	5′-GCTCATTGATCTCCA-3′	199
18	5′-GCTCGTTGATCTCCA-3′	60
19	5′-GCTCATTGACCTCCA-3′	167
	Biotinylated complementary sequences	
21B	5′-ACACCAAAGATGATA-3′-biotin	356
22B	5′-AACACCAATGATATT-3′-biotin	208
23B	5′-TCGTTGACCTCCACT-3′-biotin	396
24B	5′-TCGTTGATCTCCACT-3′-biotin	473
25B	5′-CTTGCTCGTTGACCT-3′-biotin	473
26B	5′-CTTGCTCATTGACCT-3′-biotin	459
27B	5′-GCTCATTGATCTCCA-3′-biotin	151
28B	5′-GCTCGTTGATCTCCA-3′-biotin	259
29B	5′-GCTCATTGACCTCCA-3′-biotin	225

Sequence identification number, sequence, and DNA concentrations obtained from the vendor are shown. DNA chips were constructed with 15-mer oligonucleotides to detect three mutations involved in cystic fibrosis. Note sequences 1 and 2 have little in common with sequences 3–9. Three human genetic mutations involved in cystic fibrosis are indicated in parentheses by standard notation. ΔF508 indicates a 3-bp deletion that results in removal of Phe-508 of the cystic fibrosis transmembrane conductance regulator polypeptide (31). The G → A change at codon 551 results in a change from Gly to Asp (G551D) and the C → T change results in a stop codon in place of the normal codon for Arg (R553X) (16). WT indicates the wild-type or normal sequence at each position. For each single mutation, the wild-type sequence was also present on the chip. In addition, sequence 7 contained a double mutation. While not physiologically relevant, the double mutation was included to evaluate chip performance. Complementary oligonucleotides 11–19 for ^{32}P-end-labeling and oligonucleotides 21B–29B with 3′-biotin labels for wave guide detection were synthesized.

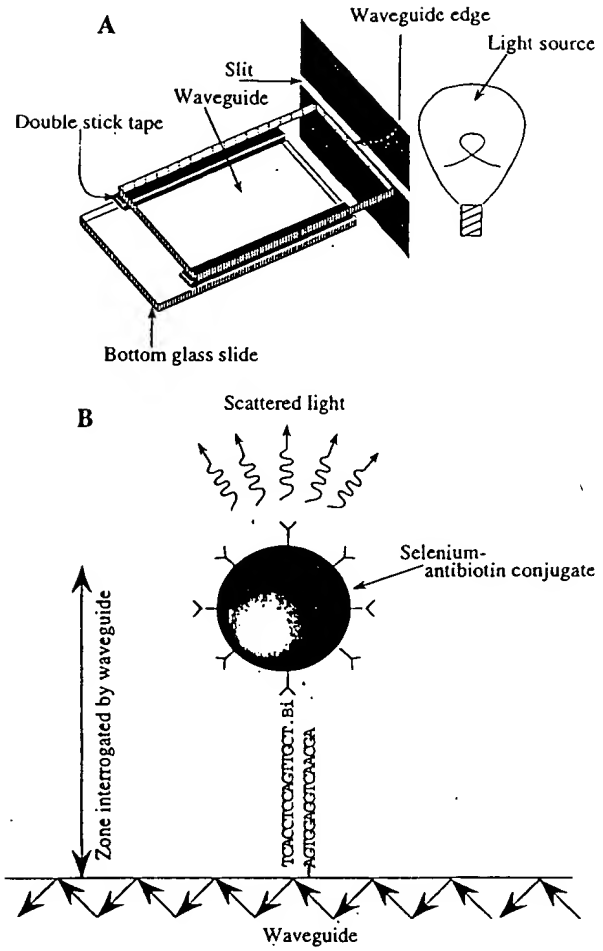

FIG. 1. (*A*) Wave guide glass slide with a bottom glass slide joined with double-stick tape to form a channel 75 μm thick and 2.54 cm wide. The DNA capture zones are placed on the upper wave guide slide. The DNA solutions or the anti-biotin conjugate are introduced into channel by capillary action. Light is injected into the edge of the wave guide and propagates by total internal reflection [zig-zag pattern (*B*), not drawn to scale!]. An evanescent wave is created on the wave guide, and light is scattered when particulate label binds to the surface. The light scattering signal is viewed/imaged from above.

conjugate was applied for 1–5 min. For the channel, the DNA solution is introduced by capillary action. The conjugate solution was then applied as a pool at one end of the channel, a paper towel was placed at the opposite end, and the conjugate solution replaced the DNA solution. When a single slide was used, 50 μl of DNA solution was applied to the chip, incubated 5 min, drained, and rinsed with PBS, and a drop of conjugate solution was applied to stain. In this case, the chips were washed and then stored in PBS in a Petri dish before imaging by wave guide illumination.

Hybridization for Autoradiography. Oligonucleotides were end-labeled with ^{32}P by reacting 0.5 μg of DNA with 5 μl of 10× New England Biolabs kinase buffer, 30 μCi of [^{32}P]ATP (3000 mCi/mmol; 1 Ci = 37 GBq; Amersham), and 20 units of New England Biolabs T4 polynucleotide kinase in a total volume of 50 μl for 30–60 min at 37°C. Hybridizations were carried out in seal-a-meal pouches with 2.5 ml of 6× SSC/0.5% SDS (6× SSC = 900 mM NaCl/100 mM sodium citrate, pH 7.0) for 16 h. The slides were washed four times with 6× SSC, dried, and exposed to Kodak XAR5 film for 1–2 h.

Wave Guide Detection. Light from a 150-W lamp (Dolan Jenner Fiberlite series 180) was injected into the end of the wave guide by use of a slit (Fig. 1*A*). Light scattering from the selenium conjugate adsorbed on the wave guide chips was observed visually and was also recorded by using a Cohu CCD camera (Cohu model 4815) operating at 30 frames per sec. Output from the camera was either fed into a video cassette recorder for later analysis or directly into an 8-bit frame grabber (Imaging Technology, PC Vision plus). The single digitized images were analyzed by using the software IMAGE MEASURE (Phoenix Technology, Seattle), IMAGEPRO PLUS (Media Cybernetics, Silver Spring, MD), or NIH IMAGE (obtained from the FTP Internet site zippy.nimh.nih.gov/pub/nih-image).

For the wave guide melting studies, an aluminum heating block, 1.5 × 1.5 × 0.25 inches (1 inch = 2.54 cm), containing two heating elements and a thermocouple was placed beneath the wave guide (Watlow 965 demonstration unit, Winona, MN).

Liquid Melting Studies. Melting temperatures in liquid were measured with a Hewlett–Packard model 8452A spectrophotometer. The single-stranded DNA was diluted to an A_{260} of 0.05–0.1 in 10 mM Tris·HCl (pH 7.8) with 1 M NaCl or 0.15 M NaCl (0.5–1 μM DNA).

RESULTS

Fig. 1*B* shows the basic principle involved in signal generation with the wave guide DNA chip. Light is injected into the end of the wave guide and propagates by total internal reflection thereby creating a uniform two-dimensional evanescent wave. The evanescent wave extends 100–300 nm into the solution above the wave guide. Thus, only the selenium particles that are in close proximity to the wave guide surface scatter light. Selenium particles are concentrated on the wave guide surface via DNA hybridization and the subsequent binding of the conjugate to biotin. Signals appear as bright spots on a darker background wherever DNA is hybridized. Experiments with 70-nm gold particles produced scattering signals that were much less intense than the 200-nm selenium particles.

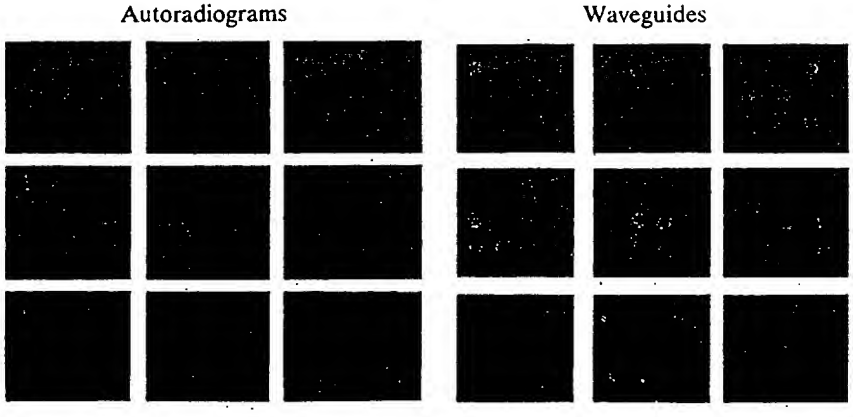

FIG. 2. Comparison of hybridization patterns obtained by optical wave guide and autoradiography. Sequences 1–9 were immobilized to form 3 × 3 DNA chips so that row 1 had sequences 1–3, row 2 had sequences 4–6, and row 3 had sequences 7–9. The chips were incubated with each of the nine complementary oligonucleotides. (*Left*) Results from ^{32}P-labeled sequences 11–19. A dark zone on a light background indicates hybridization. (*Right*) The wave guide results with oligonucleotides 21B–29B. The chips are arranged as in Table 2. The wave guide signal is a bright zone on a dark background. In this case, the wave guide chips did not have a second glass slide to form a channel and, hence, were washed with PBS and submerged in a Petri dish containing PBS for imaging.

Table 2. Theoretical melting temperatures computed for the various combinations of DNA sequences

Seq. 11 or 21B			Seq. 12 or 22B			Seq. 13 or 23B		
50	27	<0	24	47	<0	<0	<0	58
<0	<0	<0	<0	<0	<0	52	35	25
<0	<0	<0	<0	<0	<0	37	44	44
Seq. 14 or 24B			**Seq. 15 or 25B**			**Seq. 16 or 26B**		
<0	<0	49	<0	<0	35	<0	<0	20
56	20	10	25	58	52	10	49	56
41	49	33	33	41	41	38	29	46
Seq. 17 or 27B			**Seq. 18 or 28B**			**Seq. 19 or 29B**		
<0	<0	30	<0	<0	41	<0	<0	41
38	26	34	49	38	29	33	38	46
56	49	49	52	58	45	52	45	58

Theoretical melting temperatures (T_m, °C) were computed for hybridization products formed between sequences 11 and 19 or 21B and 29B and each of the nine immobilized sequences on the chip. The melting temperatures were calculated by using the equation: $T_m = 81.5 + 16.6 \log[Na] + 0.41(\%\,GC) - 675/(length) - (\%\,mismatch)$, with [Na] = 1. The computed melting temperatures are spatially arranged to correspond with the location of DNA capture zones displayed in Fig. 2.

The DNA sequences 1–9 were used to form 3 × 3 arrays of capture sites. Each of the complementary sequences were incubated with a chip and the hybridization patterns were detected by using autoradiography or the optical wave guide (Fig. 2). The patterns of hybridization were similar for the two detection methods. In most cases the wave guide signals were visible as soon as the selenium conjugate solution covered the chip. Incubation of conjugate for periods longer than ≈5 min did not result in an appreciable increase in signal intensity at any of the spots.

Only DNA complementary to zones 1 and 2 gave simple hybridization patterns. The other more complicated patterns for hybridizations 23–29 are consistent with the sequence homologies and each pattern is indicative of the particular target. By using the equation described in the program OLIGO 4.0 (National Biosciences, Plymouth, MN), the theoretical melting temperatures (33) were computed and are displayed in Table 2. There is a qualitative correlation between the theoretical melting temperatures (Table 2) and the hybridization patterns (Fig. 2). Apparently, pattern analysis of chips with multiple probes of different effective lengths, sequences, and therefore, different hybridization constants is a method of resolving targets that differ by one or two bases.

Hybridization under low-stringency conditions does not allow for discrimination of single-base mutations when analysis is limited to the wild-type and mutant probes; compare zones 3 and 4 when hybridized with sequence 23B or 24B and zones 5 and 6 when hybridized with sequence 25B or 26B. The single-base change in the middle of a 15-mer results in similar binding to both the exact matching and the mismatched zones when hybridization is carried out at room temperature.

Fig. 3 shows the results of an experiment in which a single-base change can be resolved with a two-zone chip in ≈5 min via melting. DNA chips with two spots, sequences 3 and 4, were hybridized with sequence 23B or 24B, so that in each case an exact match and a single-base mismatch was present. The wave guide scattering signals appeared in a matter of seconds after application of the selenium conjugate and stopped increasing after 2–3 min. Some of the initial increase can be seen in the data for sequence 24B (Fig. 3) as heating was initiated before signal development stopped. Since DNA was already hybridized, the initial increase in light scattering was due to the binding of selenium conjugate to the biotinylated DNA captured on the wave guide surface. As the DNA duplex dissociates, the attached selenium conjugate diffuses out of the evanescent wave and the scattering signal decreases. In each experiment, the exact matching zone (i.e., zone 3 for 23B and zone 4 for 24B) displayed the higher melting temperature and was the last to disappear. Our

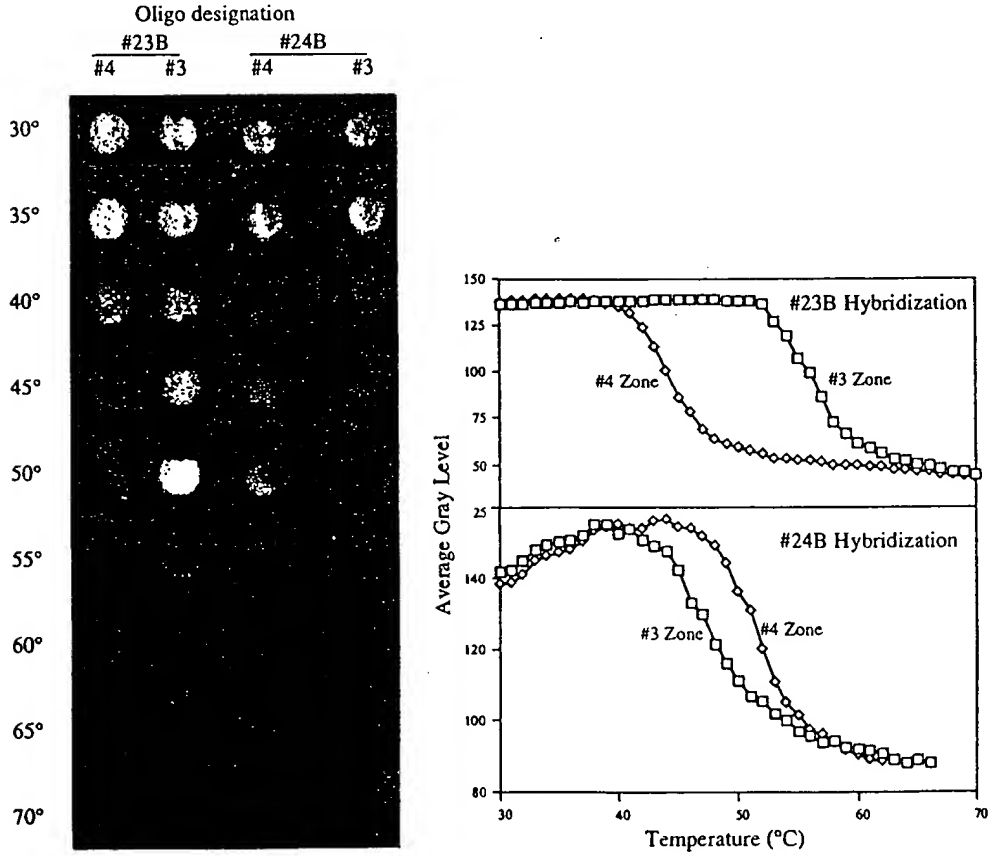

FIG. 3. (*Right*) Chips with two capture zones, sequences 4 on left and 3 on right, were incubated with sequences 23B or 24B as indicated. Selenium conjugate was then introduced into the channel and the wave guide signal was allowed to develop for 2–3 min. (*Left*) The temperature was increased and the signal intensity at each spot was measured at 1°C increments to produce the DNA chip melting curves. Images of the chip were captured at 5°C increments and are displayed next to the temperature (in °C). The selenium conjugate remained in the channel throughout the experiment.

Table 3. Effect of a single-base mismatch on melting temperature

Sequences in duplex formed	Melting temperature, °C: Liquid, 1 M NaCl	Liquid, 15.4 mM NaCl	Wave guide chip
3–13/23B	68	47	57
4–13/23B	54	34	45
3–14/24B	59	37	48
4–14/24B	66	43	52

Melting temperatures were measured in liquid by using UV adsorption and on the wave guide by using selenium anti-biotin label. Sequences 13 and 14 were used in the liquid melting experiments and the biotinylated analogs 23B and 24B were used for the wave guide melting experiments. The concentration of Na^+ in the wave guide experiment is >15 mM due to the presence of Na present in the casein; the final Na concentration is not known.

interpretation of this data is that the DNA duplex is the most thermal-labile noncovalent interaction in the system.

The melting temperature was estimated as the midpoint between the high and low points of the transition. Table 3 shows the chip results compared with the analogous experiments in liquid at two ionic strengths. The chip melting temperatures correlate with the liquid melting temperatures for 15.4 mM and 1 M NaCl conditions, albeit ≈10°C different in each case. Both the chip and liquid melting curves show a greater difference in the effect of the mismatch for the 23B hybridization than for the 24B hybridization as expected from the relatively stable mismatch.

Sensitivity of the wave guide system was estimated by incubation of sequence 23B with a DNA chip containing two sequence 3 spots and two sequence 1 spots in a cross-diagonal pattern with 0, 0.4, 4.0, and 40 nM DNA. At 0.4 nM DNA, a signal could just be seen/detected at zone 3. Except at the highest concentration, no measurable signals at the sequence 1 capture zones were observed and the zero concentration of sequence 23B was not detectable at zone 3. Digital analysis gave an average signal minus background of 7 gray levels for the 0.4 nM spot compared to ±2 gray levels for background variation in the zero concentration. Thus, 0.4 nM is an approximate limit of detection (defined by a signal >2 standard deviations above the mean background signal). It is likely that sensitivity could be increased by increasing the conjugate concentration.

DISCUSSION

The fluorescent oligonucleotide concentration that was reported in the evaluation of the Affymetrix photolithographically produced DNA chips, with confocal microscopy was 10 nM (12). A wave guide signal sufficient for single-base discrimination has been generated between 4 and 40 nM DNA and is, therefore, comparable to a fluorescence signal system. The pin/spotting method did not create a sufficiently uniform capture zone for further quantitative analysis.

The use of the optical wave guide readout has significant advantages over fluorescence readout with confocal microscopy (12), which is regarded as the current "gold standard." The readout is essentially instantaneous with real-time video imaging, as opposed to the requirement for a time-consuming scanning procedure; backgrounds are minimal as the wave guide only interrogates a boundary layer for signal generation (Fig. 1*B*), and paradoxically, high concentrations of unbound colloid have a high absorbency and, hence, actually suppress background scattering from the solution phase.

Although the initial signal develops for 2–3 min before showing saturation, it is not reasonable to assume the dissociation reaction will occur with the same kinetics. Signal generation is dominated by the diffusion of selenium conjugate to the adsorbing surface from the bulk solution (≈20 μm). In contrast, signal reduction via melting is limited only by the time required for the selenium to diffuse out of the 200- to 300-μm evanescent wave. This process has been accelerated by using forced-air heating and melting for an entire array accomplished in a few seconds with single-base discrimination maintained (D.I.S., unpublished results).

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54) Title: DETECTION OF NUCLEIC ACID POLYMORPHISM

(57) Abstract

This application describes a method of detecting DNA variation by monitoring the formation or dissociation of a complex consisting of: (a) a single strand of a DNA sequence containing the locus of a variation; (b) an oligonucleotide or DNA analogue probe specific for one allele of the variation and capable of hybridising to the single strand (a) to form a duplex; (c) a marker specific for the duplex structure of (a) plus (b) which forms a complex with the said duplex and reacts uniquely when interacting within the duplex, which comprises continually measuring an output signal indicative of interaction of the marker with duplex formed from the strand (a) and probe (b) and recording the conditions at which a change in reaction output signal occurs which is attributable to formation or dissociation of the complex and is thereby correlated with the strength with which the probe (b) has hybridised to the single strand (a). The method, termed Dynamic Allele Specific Hybridization (DASH), scores nucleotide differences in DNA sequences. Fluorescent markers are convenient as markers to underline variations in fluorescence resulting from denaturization or hybridization of the complex.

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DETECTION OF NUCLEIC ACID POLYMORPHISM

This invention relates to nucleic acid sequence variation and more particularly to the scoring of variants (polymorphisms and mutations) occurring in natural DNA sequences.

In genomes of species such as the human it is estimated that on average 1 in 10^3 nucleotides is variant between any two equivalent chromosomes. Although most such variations will be functionally neutral, a small proportion will underlie human phenotypic differences including the risk of disease. DNA variations may be investigated by determining the extent of hybridisation of allele specific probes against DNA segments containing the locus of the variation. In this way, it is possible to record 'matches' (presence of DNA identical to the probe) and 'mis-matches' (presence of DNA non-identical to the probe) for DNA samples from individuals under investigation. However, a problem with existing methods of this type is that it is difficult to determine an adequately discriminatory hybridisation stringency. An improved method of increased reliability and simplicity is therefore much needed.

The present invention comprises a method of detecting DNA variation which comprises forming a complex consisting of :

(a) a single strand of a DNA sequence containing the locus of a variation,

(b) an oligonucleotide or DNA analogue probe specific for one allele of the variation hybridised to the single strand (a) to form a duplex, and

(c) a marker specific for the duplex structure of (a) plus (b) and which reacts uniquely when interacting within the duplex,

(d) continually measuring an output signal of the extent of the resulting reaction of the marker and the duplex whilst steadily increasing the denaturing environment containing the complex, and recording the conditions at which a change in reaction output signal occurs (herein termed the denaturing point) which is attributable to dissociation of the complex and is thereby correlated with the strength with which the probe (b) has hybridised to the single strand (a).

In the assay as defined above the complex is first formed and then denaturing conditions are applied to determine the point at which the duplex dissociates. When heating is employed as the method of denaturing the duplex the reaction is carried out in the following way. The test sample is first cooled to hybridise the oligonucleotide or probe to the target DNA, and then heated steadily in a controlled and monitored fashion to detect the denaturing temperature. It will be appreciated, however, that the formation of duplex DNA is an equilibrium reaction, i.e. a two way reaction. It is therefore possible to reverse the order of the events described above. Thus all the defined components of the reaction can be brought together at raised temperature and then cooled in a similarly controlled and monitored fashion to detect the temperature at which the duplex (and complex) is formed . This may be described as the "annealing" temperature. This can be considered to be equivalent to the "denaturing" temperature, but will actually be a different value due to the complex chemistry involved. This principle of equivalence can of course be applied when denaturing/annealing conditions other than heating are employed for the purposes of the invention.

The method defined in the previous paragraph is one in which the components (a), (b) and (c) are brought together prior to formation of the defined complex and under conditions in which (a) and (b) do not hybridise, whereupon the conditions of their environment are steadily adjusted to cause formation of the duplex and resulting complex, and a reaction output signal is obtained indicative of the occurrence of hybridisation of (a) and (b) (herein termed the annealing point).

More generally, therefore, the invention comprises a method of detecting DNA variation by monitoring the formation or dissociation of a complex consisting of :-

(a) a single strand of a DNA sequence containing the locus of a variation,

(b) an oligonucleotide or DNA analogue probe specific for one allele of the variation and capable of hybridising to the single strand (a) to form a duplex,

(c) a marker specific for the duplex structure of (a) plus (b) which forms a complex with the said duplex and reacts uniquely when interacting within the duplex,

which comprises continually measuring an output signal indicative of interaction of the marker with duplex formed from the strand (a) and probe (b) and recording the conditions at which a change in reaction output signal occurs which is attributable to formation or dissociation of the complex and is thereby correlated with the strength with which the probe (b) has hybridised to the single strand (a).

The invention also comprises a method of detecting DNA variation which comprises bringing together

(a) a single strand of a DNA sequence containing the locus of a variation,

(b) an oligonucleotide or DNA analogue probe specific for one allele of the variation and capable of hybridising to the single strand (a) to form a duplex,

(c) a marker specific for the duplex structure of (a) plus (b) which forms a complex with the said duplex and reacts uniquely when interacting within the duplex,

the components (a), (b) and (c) being brought together under conditions in which :

EITHER (i) the component (a) hybridises to component (b) and the complex is formed with component (c)

OR (ii) the components (a) and (b) do not hybridise and the complex with component (c) is not formed,

thereafter steadily and progressively adjusting the conditions of the environment, respectively,

EITHER (i) to denature the formed duplex and cause dissociation of the complex,

OR (ii) to cause formation of the duplex and resulting complex,

and continually measuring an output signal indicative of the extent of hybridisation of (a) and (b) and resulting complex formation with (c)

and recording the conditions in which a change of output signal occurs which is indicative of, respectively (i) dissociation of the complex or (ii) formation of the complex.

DNA duplexes can be denatured in a number of ways. The most usual systems employed are raised pH or increased temperature. Thus a controlled steady temperature increase is used to apply denaturing 'pressure' to the duplex, to examine at which point matched and mismatched duplexes denature. As an alternative a controlled steady pH increase can be used. Additionally, the principle of the invention may admit a DNA 'micro-chip' format (sub mm scale assay areas on flat surfaces - with the potential for mounting over electrical chips) in which case it opens the possibility of the use of increased negative electric charge (charge repulsion) to push the DNA strands (also negatively charged) away from the surface. If one partner of the duplex is surface bound, this effect will tend to denature the DNA, as for pH and temperature.

In order to choose alternative signal detection methods, any system that gives a different signal for double stranded and single stranded DNAs can be used as the basis for detecting the denaturing (or hybridising) of the probe plus target DNA duplex. The most well known physico-chemical difference between double stranded and single stranded DNA is the spectrum of UV light absorption caused by these molecular species. Apparatus can be devised to utilise this parameter.

A preferred marker for use in the method defined above is one based on fluorescence. Where a fluorescent marker is used, the present invention comprises a method of detecting DNA variation which comprises forming a complex consisting of :

(a) a single strand of a DNA sequence containing the locus of a variation,

(b) an oligonucleotide probe specific for one allele of the variation hybridised to the single strand (a) to form a duplex, and

(c) a marker specific for the duplex form of (a) and (b) and which fluoresces when bound to or intercalated within the double stranded DNA,

continually measuring the resulting fluorescence whilst steadily increasing the temperature of the environment containing the complex, and recording the temperature (herein termed the melting temperature) at which a decrease of fluorescence occurs

which is attributable to dissociation of the complex and is thereby correlated with the strength with which the probe (b) has hybridised to the single strand (a).

In carrying out the method it will be usual to form a series of two or more complexes of the kind defined, each with a probe specific for a different allele of the variation, and observing their respective transition points so as to distinguish alleles of the variation plus the homozygous or heterozygous state if appropriate. Using fluorescence, the melting temperature is conveniently determined by reference to the negative or positive first or second derivative (differential) of the output signal (fluorescence measurement) curve.

The method of this invention is advantageously carried out with the single strand DNA attached to a support material, most conveniently by a biotin/streptavidin type interaction. The single strand is derived from a double stranded DNA product of PCR amplification of a target sequence. Low complexity sequences, such as cultured viral genomes, purified cloned DNAs etc. can be end-labeled with streptavidin or such and used without specific amplification. It is convenient to work with a PCR product over 100 base pairs or preferably from 40 to 100 base pairs in length. The complex may be formed by adding the probe and marker to the single strand in an appropriate buffer solution.

As indicated above, the assay may be performed with the target DNA bound to a surface. However, the invention is not limited to any one format. Having both target and probe in free solution is also possible. There is also the option of localising both to some shared region. Since the method involves forming a duplex between the two species, if neither or only one is localised, then one has to rely upon diffusion to bring the molecules together. However, by localising the species in mutual close proximity (e.g. both to a surface, or even joining the two together at their ends) the efficiency of the duplex formation can be increased. This is of particular importance if hybridisation is

used as the basis of the assay rather than denaturation. It will also improve the speed of the initial hybridisation for the denaturation based format. We have applied this for directly linked target and probe sequences i.e. one joined to the end of the other with a 'stuffer' sequence of irrelevant DNA between them to constitute a 'hinge'.

The method of the present invention it is not restricted to the use of single oligonucleotide probes. Additional probes can be used which anneal sequentially along the target DNA sequence. These might or might not be placed immediately contiguous i.e. with no gaps. Contiguously located probes will co-stabilise each other by the chemistry of 'base stacking' which is a well established phenomenon. It is also possible to construct a direct physical link between the adjacent probes. All the extra duplex DNA resulting from these extra probes increases the signal level of the assay.

1. Principle of the Method

The method of the invention, termed Dynamic Allele Specific Hybridization (DASH) is a technique that detects and scores single nucleotide differences in DNA sequences. In this assay, one strand of a double stranded DNA (e.g. a PCR product) is bound to a solid surface, and the other strand is removed. An oligonucleotide probe, specific for one version of the variation (an allele), is allowed to hybridize to the bound single strand. Next, an intercalating dye is added which fluoresces specifically in the presence of double stranded DNA (i.e., the oligonucleotide probe hybridized to the DNA sample). The reaction is now heated at a steady rate through a range of temperatures, while continually measuring fluorescence. As the temperature rises, the fluorescence decreases gradually until a temperature is reached where the oligonucleotide probe dissociates from the target DNA. This temperature is known as the melting temperature, or Tm. At this point, there is a rapid decrease in fluorescence.

DNA variations can have two or more alleles. To determine which alleles are present in a given DNA sample, an allele specific probe for each version of the variation may be

assayed against the DNA sample. By comparing the 1st derivative of the fluorescence data from the two probings, it is possible to determine whether one or both alleles are present in the DNA sample.

2. Methodology

In outline form, the method consists of the following:

1. PCR amplification of the test DNA sequence
2. Binding one strand of the PCR product to a surface
3. Elution of unbound DNAs and PCR components
4. Neutralization of pH
5. Hybridization of an Allele Specific Oligonucleotide
6. Removal of excess Allele Specific Oligonucleotide
7. Detection of fluorescence during a heating regime

(7a. Repetition of steps 4-7 for alternative allele probes)

8. Analysis of fluorescence outputs

2.1. PCR amplification

To test genomic DNA, Polymerase Chain Reaction (PCR) is used to amplify a segment of DNA containing a known variation. Ideal conditions involve amplifying a short PCR fragment (from 40-100bp), with 18-30 nucleotide long primers. One primer is biotinylated at its 5' end, allowing binding to a solid surface in a later step. Taq Gold, or other "Hot Start" type PCR conditions are used to limit primer dimer artefacts as much as possible. Effective PCR buffer conditions are as follows, with cycle times and numbers appropriate for the particular DNA fragment in question:

Primer 1 (non-biotinylated)	100ng
Primer 2 (biotinylated)	50ng
DMSO	5%
Nucleotides	5.0 nmoles each dNTP

PCR Buffer	to 1x
Enzyme (e.g. Taq Gold)	0.75 units
Water to a total volume:	25 µl

To limit the amount of non-incorporated biotinylated primer (which competes for binding sites on the solid surface), it was determined that 20ng of biotinylated primer is sufficient. With a ratio of 20ng biotinylated primer to 100ng of non-biotinylated primer, the PCR product formation is still efficient, and the lower concentration of biotinylated primer decreases competition for streptavidin sites when binding to the solid surface.

PCR products of longer lengths (over 100 base pairs) work also, but there are some considerations. With longer PCR products, the variation should be located towards the biotinylated primer as there will be less kinetic motion at this end. Secondary structures can inhibit efficient binding of the probe and should be avoided. Also, the binding efficiency of long PCR products to the plates is reduced, presumably associated with secondary structure complications, as well as slower kinetics of molecule diffusion.

Short PCR products (40-100bp) are preferable for several reasons. Less primer dimer artefact is seen with short PCR products. In addition, the overall efficiency of PCR is often superior when amplifying short products. If the binding capacity of the solid surface can be increased sufficiently, multiplex PCRs can be considered for use in the DASH assay. The short PCRs assists both in the efficiency of the multiplex PCR, and in the binding to the surface.

2.2. Binding one strand of the PCR product to a Solid Surface

The current binding surface format used is a 96 well microtitre plate that has been coated with steptavidin (available from various manufacturers). The total volume (25µl) from one PCR is placed at room temperature in a well of the streptavidin coated plate, along with 25µl buffer I (see sections 2.2.1-2.2.3 for buffer descriptions). The PCR

product then becomes physically attached to the plate via the biotin label on the PCR product binding to the Streptavidin coating on the plate. Binding is left to proceed for anywhere between 5 minutes to 24 hours. Since the binding is 90% complete within one hour, the maximum efficiency is usually achieved after 30 minutes to 2 hours. Typically, less than 20% of the PCR product becomes bound to the plate, even at maximum efficiency. The binding solution can thus be removed and placed in a second well, which will also be completely saturated.

It is important in this step, as with all the following steps, that there are no air bubbles in the reaction tubes (wells on the microtitre plate). Air bubbles interfere with reactions between the solution and the surface of the microtitre plate, and should be removed before each incubation step. This can be done with a pipette tip, or tapping the well with a finger. In addition, it is necessary to remove as much as possible of the volume of solutions from the reaction tubes between steps. The reaction tube should appear "empty" before proceeding to the next step, with no visible solution left in the bottom of the well.

2.2.1. Hepes buffer (buffer I)

Buffer I consists of 100mM Hepes, 50mM NaCl, 10mM EDTA, pH 7.8. There is an important reason why this buffer was chosen. It tends to dramatically standardize/normalize Tms based on the oligonucleotide length, regardless of G+C content. For example see the data given for Figure 7.1.4. Many other buffers allow sufficient allele discrimination, however the absolute Tm's observed will vary greatly depending upon the G+C content.

2.2.2. Alternative Buffers & pH

SSPE, SSC, TEN, TES, MES, and Phosphate buffers were tested, and all maintained the integrity of the experiment. The different buffers vary the observed Tms of oligonucletide/target DNA duplexes. The relationship between the Tm's of the matched

oligo probe to a DNA target compared to the mismatched oligo hybridized to a target remained fairly constant at ~8-10°C. The above buffers supported a pH range from 6.0 to 9.8. There were no significant pH affects detected in this range. Below pH 6.0, the DNA analyzed tended to degrade, and at pH 10.0 and above the Tm was decreased to close to room temperature, preventing effective fluorescence measurement.

2.2.3. Buffer/Salt Concentrations

For the above buffers, a range of buffer and NaCl concentrations (1mM to 400mM) have been tested. At low salt concentrations, the observed Tm decreased as the buffer concentration increased. For example, in Hepes buffer at concentrations 1, 10, 40, and 100mM with 0.0M NaCl, The Tm values decreased from 88°C, 84°C, 78°C, and 72°C respectively in one experiment.

At NaCl concentrations above 10mM, the salt concentration rather than the buffer concentration becomes the major factor affecting the Tm values. Like buffer concentration, increasing NaCl concentration has the tendency to decrease the Tm. A range of NaCl concentrations were tested from 1mM to 800mM. At concentrations above 200mM, the data becomes difficult to interpret (the curves were marked by random fluctuations).

2.2.4. Plastic-ware

The microtitre plates and tubes employed must be made from fluorescence free plastic, and thus provide no additional background to the assay. The microtitre plates and tubes are also frosted to eliminate any fluorescence that may be detected from the outside of the tube. Thus, the plate and caps offer virtually no background to the experiment. Almost all background fluorescence is accounted for by the physical apparatus (excitation/detection equipment).

2.3. Elution of second strand of the PCR product

Once the PCR product has bound to the plate, the non-bound PCR reagents are aspirated away, and 50µl of NaOH solution is added. This denatures the PCR product, leaving single stranded DNA attached to the plate via the biotin/streptavidin interaction. 0.05M NaOH was determined to be the minimum concentration of NaOH needed to reliably denature the double stranded PCR product, however 0.1M allows room for concentration inaccuracies, and does not interfere with subsequent reactions. We allow 0.5-5.0 minutes for full denaturation of the PCR product in 0.1M NaOH. Longer times are not necessary, but have no deleterious effects. The elution solution is aspirated away to remove all residual PCR components (non-incorporated primers, nucleotides, the enzyme, etc.), as well as the non-biotinylated PCR product strand.

2.4. Neutralization of pH

A solution of buffer I is prepared including a fluorescent dye specific for double stranded DNA. We currently use 'SYBR Green I' dye. The optimal concentration of this dye in the DASH assay is 1:10,000. Slight variations in dye concentrations do not affect the results. However, there are some characteristics of the SYBR Green I dye that should be noted (see sections 2.7.2., 3.1. and 3.2.). 50µl of the Buffer I/dye solution is then added to the reaction tube. This buffer will both neutralize any remaining NaOH, and serve as the hybridization buffer in the next step. There should be minimal delay time before proceeding to the next step to minimise the opportunity for formation of secondary structures in the test DNA molecules.

2.5. Hybridization of an Allele Specific Oligonucleotide Probe

The SYBR Green I dye is included in the hybridization solution of the last step as it stabilizes the interaction between the oligonucleotide probe and the test DNA. 30pmols of oligonucleotide probe (in 1µl volume) is added to the reaction tube. The probe can alternatively be added as part of the neutralization buffer. This amount of probe allows hybridization to be completed within seconds, therefore there is no practical minimum

incubation time for this step. Optimal results are obtained by performing a heating plus cooling step (heating to greater than 50 ^{o}C and cooling steadily over ~15 minutes to room temperature), rinsing the wells clear of unbound probe, and re-filling with 50µl of the Buffer I/dye solution. Lower amounts of probe necessitate longer incubation periods, while higher concentrations do not decrease the necessary time for annealing. For further probe details, see section 2.5.1.

2.5.1. Probe Design and Use

Probe lengths of 13-25 base pairs have been tested, on three different variant loci. Allele discrimination is possible at all these lengths, however the optimal probe length was determined to be 15 basepairs. The 13mer probes denatured at temperatures close to room temperature, and were determined to be non-ideal for this technique. The 25mer achieved high fluorescence intensity (as fluorescence is a function of double strand DNA length), but allele discrimination was minimized. The 15mer probe allowed sufficient fluorescence intensity and high discrimination between alleles.

The position of the variation in the hybridization probe was also examined. With a 15mer probe, it was determined that the variant position gives the best discrimination when located in the central third of the probe. For single base variations the variant position is best placed at the central position. If the variant position is moved two bases from the centre, the assay is less discriminatory.

In order to rapidly hybridize the probe to its DNA target on the solid support, 30pmols was determined to be effective. This necessitates removal of the probe before fluorescence detection. An alternative was tested involving much lower amounts of probe (1-5pmol) for hybridization, and subsequent processing without removal of the excess probe. Although allele distinction was achieved, the fluorescence values were low and the results were highly variable. With a higher binding capacity on the plate, this strategy may prove effective, decreasing the number of steps involved in the assay.

2.6. Removal of Excess Probe

The hybridization solution is next aspirated away to remove unhybridized probe molecules, and 50µl of the buffer I/dye is added. The experiment is then ready for heating and fluorescence detection.

2.7. Fluorescence Detection and Heating Regime

The microtitre plate is placed in a heater/detector apparatus. Several devices are available which allow coincident temperature modulation and fluorescence detection as required to produce melting temperature profiles. These include a purpose-built "DASH machine" from Hybaid which allows automated scoring of alleles, and the Perkin-Elmer 7700 (Taqman) machine which was used for generation of the data presented in this document. The sample plate is heated from ~25°C to ~90°C, while continually monitoring fluorescence. Most samples denature around 65 ^{o}C $\pm$10 ^{o}C. Heating rates may vary at least between 0.01 to 1.0 ^{o}C per second with little loss of allele discrimination. We typically run assays at a rate of 0.1 ^{o}C per second. For details regarding the hardware of the Taqman device see section 2.7.1.

2.7.1. Perkin Elmer 7700 (Taqman) Sequence Detector

The detection device must detect the emission spectra given off by the double strand DNA specific fluorescent dye and keep track of the temperature at which the fluorescence data points were extracted. The excitation light source frequency must correspond to the requirements of the dye used in the DASH assay. For example, for dyes such as SYBR Green I excited near the 488nm frequency, an Argon laser or a halogen lamp (filtered for the 488nm frequency) is sufficient to excite the dye molecules. The Taqman is equipped with an argon laser that excites the fluorescent molecules. A filter is in place, removing all other wavelengths in the argon laser spectra, except for the 488nm wave length.

The CCD camera on the Taqman detects a frequency range between 500 and 660nm. The fluorescent signals are recorded into 5nm "bins". Thus "bin1" would contain the fluorescence data from 500-505nm. With the current arrangement, the light frequency range we use for the DASH assay is bin9 (545-550nm), though this is not the only bin that is effective.

2.7.2. Fluorescent Dye

The dye employed must have a specificity such that its fluorescence in the presence of double stranded DNA is at least 10 times greater than when in free solution. We currently use 'SYBR Green I' dye which has a specific signal increase of ~1000 fold.

The optimal concentration of SYBR Green I dye depends directly on the amount of DNA present in the sample. We refer to this as the 'SYBR Green effect'. If the amount of dye used is below or above the optimal concentration for the given amount of DNA, the overall fluorescence observed in the assay will be reduced. For this assay, the optimal dye dilution is 1:10,000.

Alternative dyes have also been tested, and Vistra Green (Amersham) appears to have near identical properties to SYBR Green I, and could be used as an alternative dye for the DASH assay. Other dyes, such as acridinium orange and ethidium bromide gave high background fluorescence and are therefore not suitable for DASH. Other dyes, such as Yo-Pro I and To-Pro I have not been assayed due to the inappropriate light source plus filter combination in the Taqman device.

2.7.3. Assay Solution Additives

An array of different additives were screened for effects on the assay. Common destabilizing agents, like formamide, were screened and shown to be non-beneficial to the assay. In addition, hybridization reagents like Tetra-methyl Ammonium Chloride (TMAC), Bovine Serum Albumin (BSA), and Dextran Sulphate were tested, and again

found to cause irregularities in the assay. Ionic detergents, even in trace amounts, destroyed the fluorescent signal completely. Non-ionic detergent showed no negative effects, except for the tendency to produce bubbles in the reaction tubes. With non-ionic detergents, much more care was needed to make sure bubbles did not remain in the reaction tubes at the various steps in the DASH assay. Dimethyl Sulfoxide was the only additive that was found to be beneficial, in that it can be added at a 50% level or less to decrease the observed Tm values.

2.8. Analysis of fluorescence outputs

Interpretation of the output fluorescence versus temperature graphs is conveniently achieved as follows. A graph of the primary data is used to determine general information regarding how well the assay performed, i.e. the level of fluorescence and which samples may have failed. To score alleles, the results of a series of samples (different DNAs hybridized with the same allele specific probe) are plotted together according to the negative of the first derivative of the fluorescence values. For convenience, DNAs of known genotypes can be included in the series. For two alleles, two distinct peaks should be observed on the graph. These peaks correspond to maximal rates of fluorescence decrease (denaturing probe/target duplexes) in the primary data. The two peaks thus correspond to the Tms of the probe/target "matched" and "mismatched" duplexes.

The Tm peaks will be separated by at least 8°C. The higher temperature Tm peak indicates the presence in a given DNA sample of the sequence corresponding to the allele specific probe used in the experiment. This can be termed a 'match', as the allele specific probe matches perfectly to molecules in the test DNA. The lower temperature Tm peak indicates a 'mismatch', i.e. the presence of hybridising sequences in the test DNA that are similar but non-perfectly matched to the allele specific probe used in the experiment. For the typical case of a two allele system, this 'mismatch' will be the allele

not represented by the probes' sequence. Often a single sample will give both peaks, indicating that it is heterozygous for the two tested alleles.

The bound DNA samples may be reprocessed through steps 4-7 of the procedure using a probe comprising the second (or several subsequent) allele sequence(s), and the data is analyzed as above. By comparing the two sets of data, it is possible to determine with high reliability which alleles are present in the DNA samples. If a DNA sample scores a 'match' with the probe specific to allele 1, and a 'mismatch' with the probe specific to allele 2, the DNA sample is scored 'homozygous allele 1'. If the DNA sample scores a 'mismatch' with allele 1, and a 'match' with the allele 2 probe, then the sample is scored 'homozygous allele 2'. If the DNA sample is scored a 'match' for both alleles, then the sample is scored 'heterozygous for alleles 1 and 2'. For examples of primary data, 1st derivative, and 2nd derivative graphs, see section 7.1.

3. Novel Discoveries

3.1. Melting Temperature (Tm)

In this assay, the temperature at which the oligonucleotide probe disassociates from the DNA target is determined by interactions between the dye, the buffer, and the salt concentrations. SYBR Green I dye stabilizes the probe/DNA duplex structure, raising the Tm with increases in Dye concentration. Increasing Salt and Buffer concentrations decrease the Tm, presumably by decreasing the potential of the dye to bind(and so stabilise) the duplex DNA structure.

3.2. Dye effects

SYBR Green I intercalates into double stranded DNA structures and thereby increases the Tm distinction between matched and mismatched duplexes. The Tm difference between a completely 15mer oligonucleotide probe hybridized to it's perfectly matched DNA target compared to a target mismatched at the central position is roughly 8°C. The

expected difference based on melting temperature calculations for normal DNA solutions would be around 2-3°C (depending on the DNA nucleotide sequence of the probe). The current DASH format thus optimises the potential for allele discrimination.

SYBR Green I also produces an effect wherin fluorescence is dependant on the double stranded DNA concentration and the concentration of the dye. Thus, for a constant DNA concentration, titrating SYBR Green Dye levels increases the fluorescence signal until a point is reached whereafter the fluorescence signal will decrease. We call this the 'SYBR Green Effect'. The concentration of dye used in this assay (1:10,000 dilution) is optimal for the amount of DNA that can be bound to the typical microtiter plate wells at this time.

3.3. Hepes Buffer

Hepes, as the base in the hybridization buffer, has some unique characteristics in this assay. In this buffer and dye combination, the Tm of the probe/target DNA complex is a function almost solely of the length of the probe. Variations in the DNA sequence context, or the G+C content, will not alter the observed Tm. This is of extreme importance for robustness of the DASH assay.

3.4. Dimethyl Sulfoxide (DMSO)

DMSO can be added to the hybridization buffer to lower the Tm of the oligonucleotide probe/target DNA complex without compromising the integrity of the assay. The sole affect is to lower the Tm of both allele Tm's without affecting the Tm difference between them.

3.5. Ionic Detergents

Use of ionic detergents, such as Sodium Dodecyl Sulphate, as low as 0.1% concentration, will completely destroy the fluorescent signal, presumably by interacting with the dye.

4. DASH Assay Components

4.1. Dyes

SYBR Green I Dye from Molecular Probes.

4.2. Plates

Microtitre plates from various suppliers. Micro Amp Optical plates, caps, and tubes from Perkin Elmer are designed especially for fluorometric measurements.

4.3. Oligonucleotides

PCR primers and allele specific oligonucleotide probes from Interactiva Biotechnologie, all HPLC purified in order to ensure maximal quality.

4.4. Fluorescence monitoring device

The Hybaid DASH system (Hybaid Limited, UK); ABI 7700 (Perkin Elmer; used in assays presented) or other detection temperature controlled device.

4.5. Buffers

All the components of buffers were from Sigma.

4.6. Software

The software developed to analyze the raw data, first derivative, and second derivatives of sample data was written by Kin-Chun Wong (Uppsala).

5. An Alternative Assay Format

An alternate format of binding the oligonucleotide probe to the plate followed by hybridization of the PCR product should be possible, but several technical problems arose with this design when tried. First is the problem of hybridising the double stranded PCR product to the bound probe. Simple heat denaturation of the PCR product followed by cooling in the probe coated assay wells is insufficient. Presumably this is because of

displacement reactions, i.e., the PCR product reforms it's double strand and displaces any probe hybridized to the target sequence.

To eliminate the complications caused by displacement reactions, we attempted to generate single stranded DNA from the PCR product for hybridization. For this we used unequal amounts of the two primers in the PCR, thus theoretically producing an excess of one strand of the PCR product (asymmetric PCR). This did improve the assay, but only to a small degree. Importantly, optimal PCR conditions varied compromising the robustness of the DASH assay.

The long length of the PCR products in free solution also presents a problem. This could slow reaction kinetics, and potential secondary structures would interfere with the hybridisation reaction. In addition, once the long molecule is hybridized to the short fixed probe, there appears to be premature disassociation of the DNA/probe complex as thermal energy is added. In this format, it was found to be impossible to distinguish between alleles because all fluorescence signal is lost at very low temperatures. This is probably due to the hybridized PCR fragment having long non-hybridized tails sticking out into the solution. As the temperature rises, the long molecules will be pulled off by the solution kinetics rather than denatured according to Tm properties of the hybridized duplex.

Optimisation of the probe bound format will require further experimentation, involving PCR conditions, buffer components, annealing strategies, as well as other parameters. The problems concerning production of single stranded DNA molecules for hybridization, and the kinetic considerations are avoided by using the DNA molecule bound format. The non-biotinylated strand is simply eluted away for single strand DNA production, and the kinetic limitations involving premature displacement are not observed.

6. Worked Example of DASH Analysis : Detection of allelic versions of a bi-allelic single nucleotide polymorphism in the human NDUFB4 gene

A DASH experiment was performed on a single nucleotide polymorphism in the human NDUFB4 gene which is located on an autosome. This is illustrated in figure 1 as 'DNA sequence 1' and it comprises a bi-allelism between 'A' and 'G' nucleotides. Three human genomic DNA samples, X, Y and Z, were employed that were known from earlier sequence analysis to be homozygous for the 'A' allele, homozygous for the 'G' allele, and heterozygous for these alleles, respectively.

6.1. Polymerase chain reaction (PCR)

PCR was performed on 50ng aliquots of DNA samples X, Y and Z, using the PCR primers presented figure 1 as 'DNA Sequence 2' and 'DNA Sequence 3'. Reaction conditions were as follows; 25ml total volume comprising 20ng 'DNA Sequence 2' primer, 100ng 'DNA Sequence 3' primer, 0.75u AmpliTaq-Gold polymerase (Perkin-Elmer), 10% dimethylsulphoxide, 1x Perkin-Elmer PCR-buffer (including 1.5mM $MgCl_2$) and 0.2mM each of dGTP, dATP, dTTP and dCTP. Thermal cycling employed a TouchDown™ Temperature Cycling Device(Hybaid Ltd) and the following cycle conditions: 1x (10 minutes at 94°C, 30 seconds at 50°C, 30 seconds at 72°C), 17x (15 seconds at 94°C, 30 seconds at 50°C, 30 seconds at 72°C), 18x (15 seconds at 94°C, 30 seconds at 50°C, 1 minute at 72°C). This produced 48 base pair long PCR products that spanned the polymorphic locus and possessed a biotin moiety on the 5' end of one DNA strand.

6.2. Binding PCR products to a microtiter plate

PCR reaction products were mixed with an equal volume of Buffer I (100mM Hepes, 50mM NaCl, 10mM EDTA, pH 7.8) and transferred to individual wells of a streptavidin coated thin wall microtiter plate (Boehringer Mannheim). This was left at room temperature for 1 hour.

6.3. Elution of unbound strand

All liquid volume (containing non-bound DNAs and other reagents) was thoroughly aspirated from the microtiter plate. Without delay, the wells were refilled with 50ml 0.1M NaOH and left at room temperature for 5 minutes. The NaOH solution including the non-biotinylated DNA strand (now denatured from the bound strand) was then thoroughly removed.

6.4. Neutralization of reaction sample

Without delay, the wells were refilled with 50ml Buffer I including SYBR Green I dye (1:10,000 fold dilution).

6.5. Hybridization of the first allele specific oligonucleotide probe

30pmol of 'T Probe' (presented figure 1 as 'DNA Sequence 4') was added to each well in a volume of 1ml water. Optical caps (Perkin Elmer) were used to seal each well, and the plate and its contents were heated to 60 ^{o}C and cooled steadily over ~15 minutes to room temperature. This was achieved upon a TouchDown™ Temperature Cycling Device (Hybaid Ltd).

6.6. Removal of unbound probe

The optical caps were removed, and all liquid volume was thoroughly aspirated from the microtiter plate. The wells were then refilled with 50µl of Buffer I including SYBR Green I dye (1:10,000 fold dilution), and the optical caps were replaced.

6.7. Signal detection procedure

The microtitre plate was placed into a Perkin Elmer 7700 (Taqman) device, and a heating phase applied involving traversing from 35 ^{o}C to 80 ^{o}C at a steady rate of 0.1 ^{o}C per second. During this heating phase, the Taqman device repeatedly excited the samples with an argon laser light source (filtered at 488nm) and collected the

fluorescence that was emmitted at a frequency range of 545-550nm. Data points were collected at 7 second intervals for every well.

6.8. Reprobing with a second allele specific probe

Steps 3-8 above were repeated, this time replacing the 'T Probe' used in step 5 with the 'C Probe' (presented figure 1 as 'DNA Sequence 5').

6.9. Data analysis and interpretation of results

Primary data was plotted on a fluorescence data versus time graph for all wells and for both probe interrogations. This data was 'smoothed' by plotting average fluorescence values determined from a sliding window of 8 data points. The resulting 'primary' data is shown in figures 2a and 2b . A negative differential (derivative) curve of this graph was then plotted and this is shown in figures 3a and 3b. A differential curve of the negative first differential curve was then plotted to give the second derivative shown in figures 4a and 4b.

In figures 3a and 3b, high (H) and low (L) temperature peaks can be seen indicating points of maximal rate of denaturation. These represent DASH signals for matched and one-base mismatched probe-target DNA duplexes respectively. In the second differential (figure 4) these points can be inferred from the points at which the curves cross the X axis. They are also visible in the primary data (figure 2), but can be hard to discern in this representation of DASH results.

Samples X and Y are seen to have only one matched (high temperature) peak in one negative first differential graph and only one mismatched (low temperature) peak in the other negative first differential graph. This indicates that they are homozygous samples. The probing during which X and Y gave a high temperature (matched) peak indicates which allele they contain. Thus, since X gave a high temperature peak with the 'T

Probe', it is an 'A' allele homozygote. Conversely, Y gave a high temperature peak with the 'C Probe', and so is a 'G' allele homozygote.

Sample Z behaved differently to samples X and Y. It gave both high and low temperature peaks with the 'C Probe', and high and low temperature peaks (merged due to proximity into a single wide peak) with the 'T Probe'. Thus, this DNA sample must have both probe allele complementary sequences present within it. Hence it can be deduced to be a heterozygous sample containing both the 'A' and the 'G' alleles.

Figure 1. DNA sequences for use in a DASH assay for scoring alleles of a human NDUFB4 gene polymorphism

DNA Sequence 1 (Sequence listing SE1)

5':

CTGCATTTTGGCACAACCCACC(G/A)TACAACTGACAAACAGGAATGAAAC

:3'

This is a 48 base pair genomic DNA sequence representing a portion of the human NDUFB4 gene. A bi-allelic single nucleotide polymorphism (G to A) is shown in parentheses towards the centre of the sequence.

DNA Sequence 2 (Sequence listing SE2)

5': (Biotin)-CTGCATTTTGGCACAACCC :3'

This is a 19 base oligonucleotide sequence designed for use as 'PCR Primer 1' in a DASH assay for detection of alleles of the polymorphism shown in DNA sequence 1. It carries a biotin moiety attached to the 5' end.

DNA Sequence 3 (Sequence listing SE3)

5': GTTTCATTCCTGTTTGTCAGT :3'

This is a 21 base oligonucleotide sequence designed for use as 'PCR Primer 2' in a DASH assay for detection of alleles of the polymorphism shown in DNA sequence 1.

DNA Sequence 4 (Sequence listing SE4)

5': AGTTGTACGGTGGGT :3'

This is a 15 base oligonucleotide sequence designed for use as the 'C Probe' in a DASH assay for detection of the 'G' allele of the polymorphism shown in DNA sequence 1.

DNA Sequence 5 (Sequence listing SE5)

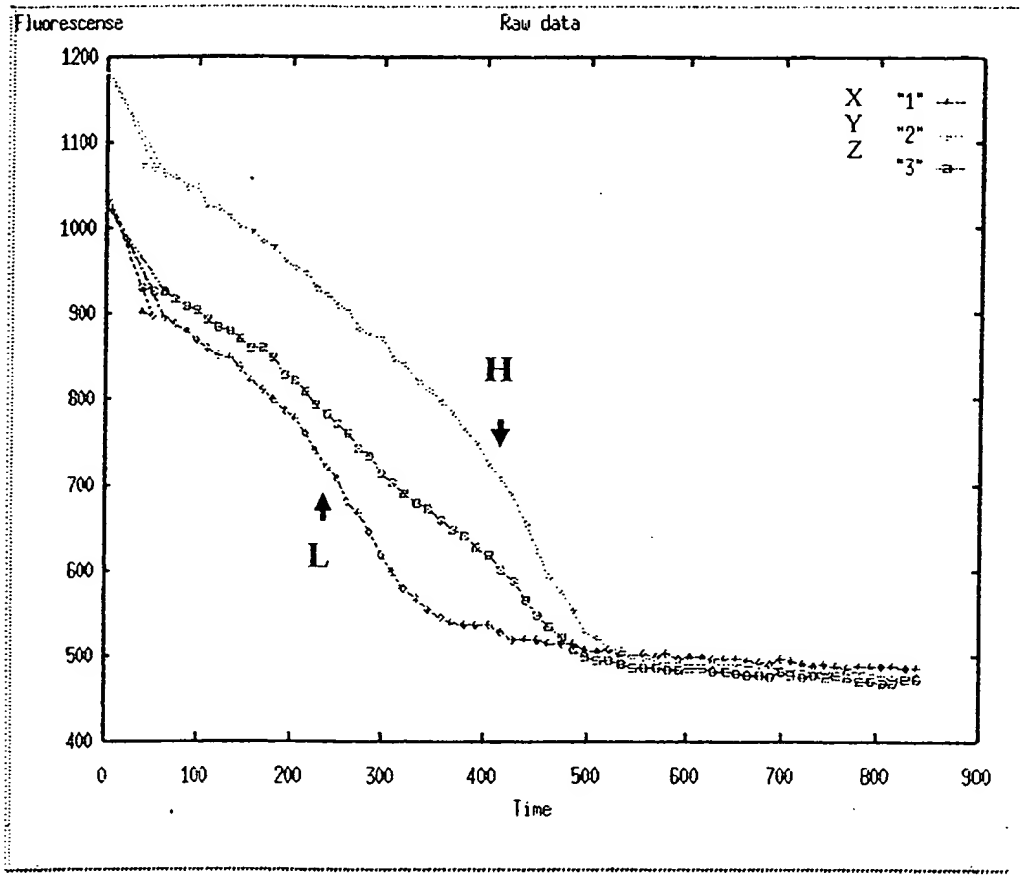
5': AGTTGTATGGTGGGT :3'

This is a 15 base oligonucleotide sequence designed for use as the 'T Probe' in a DASH assay for detection of the 'A' allele of the polymorphism shown in DNA sequence 1.

Figure 2. Primary DASH assay data for an NDUFB4 gene polymorphism

('H' and 'L' indicate points of maximum denaturation rates for matched and mismatched probe-target duplexes respectively. X, Y, and Z are the sample DNAs).

a. Results for the 'C Probe'



b. Results for the 'T Probe'

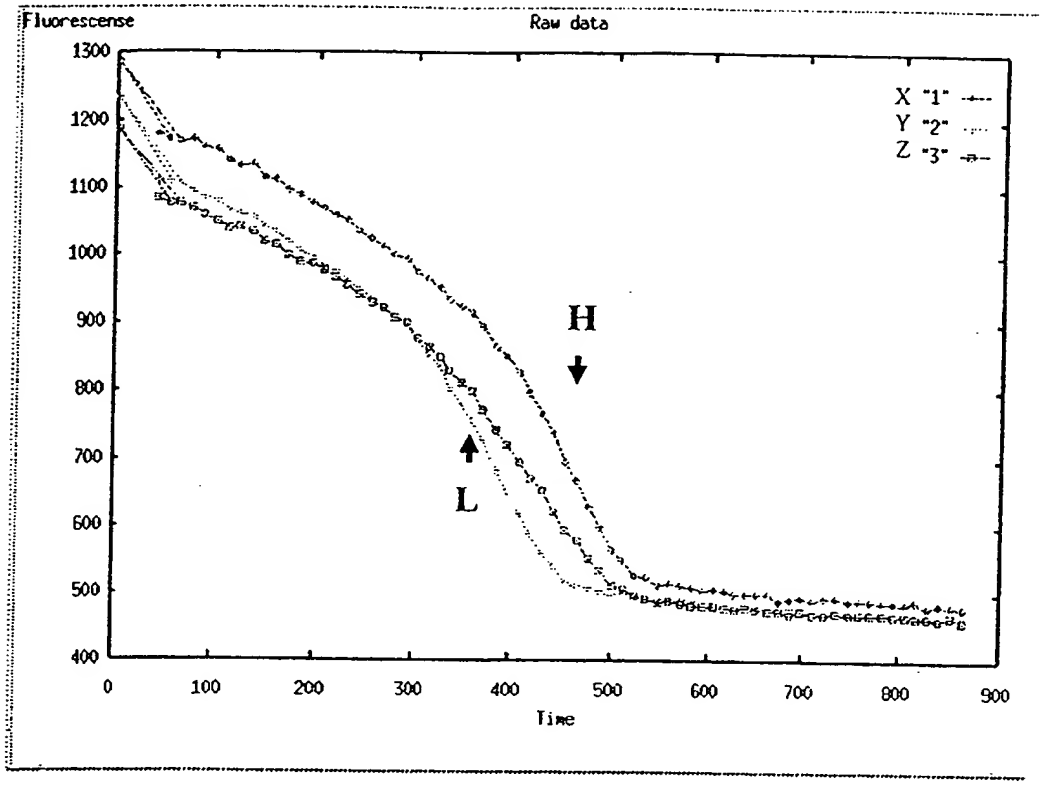
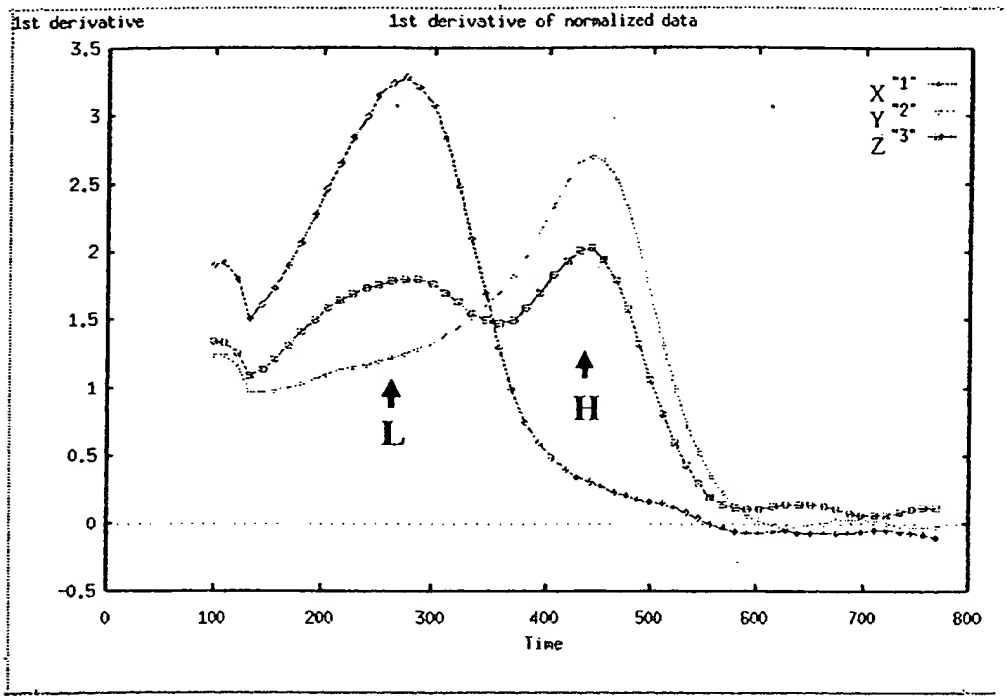


Figure 3. Negative 1st derivative DASH assay data for an NDUFB4 gene polymorphism

('H' and 'L' indicate points of maximum denaturation rates for matched and mismatched probe-target duplexes respectively. X, Y, and Z are the sample DNAs).

a. Results for the 'C Probe'



b. Results for the 'T Probe'

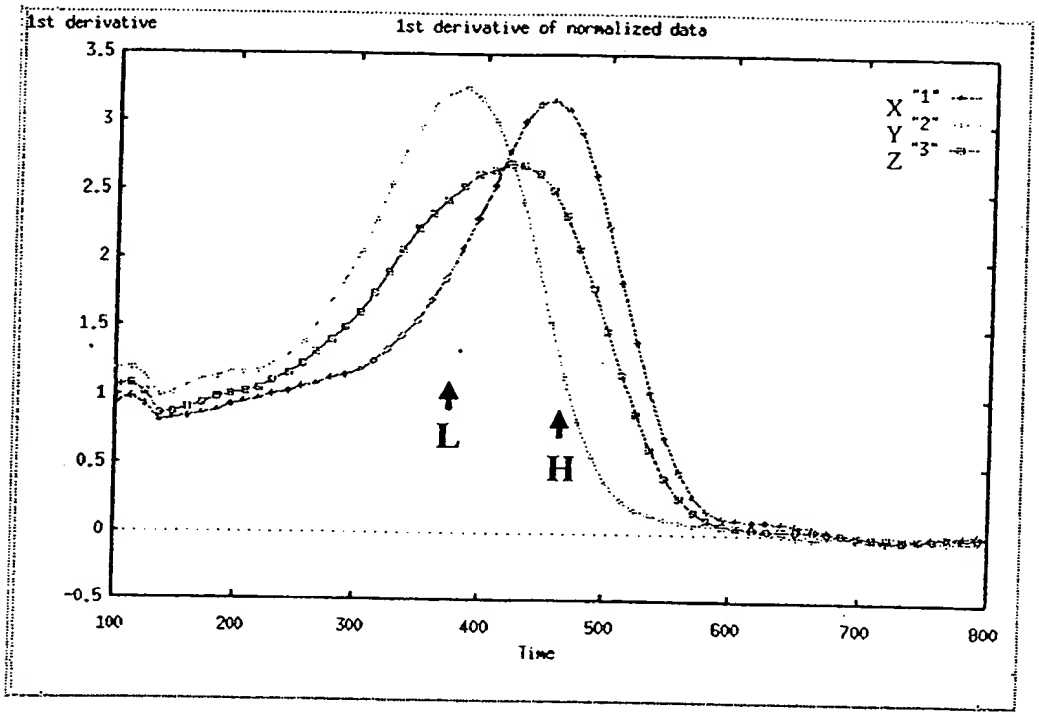
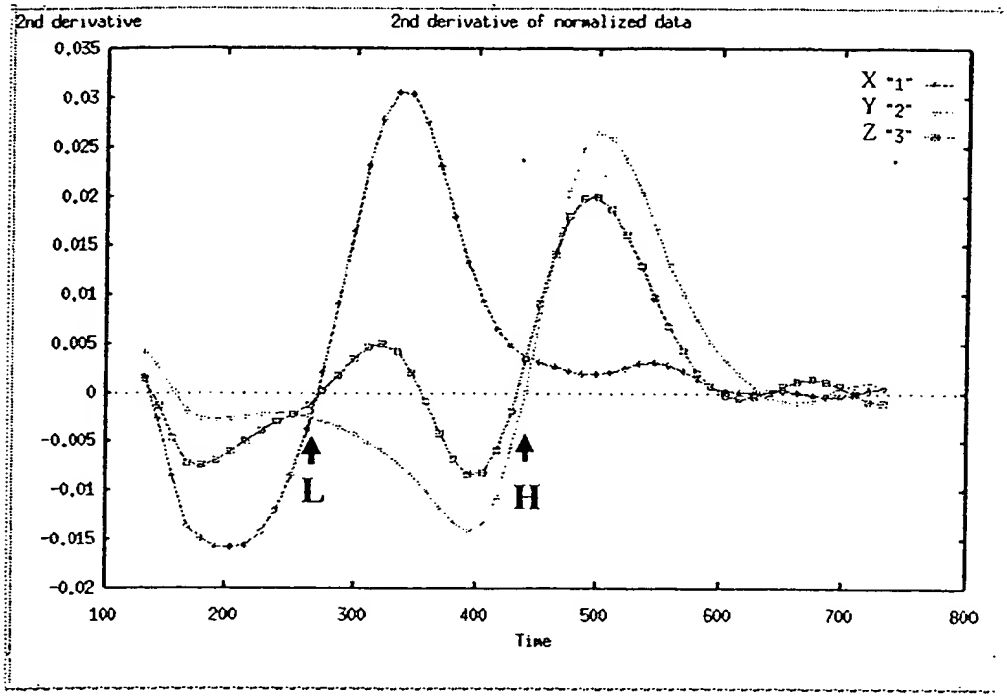


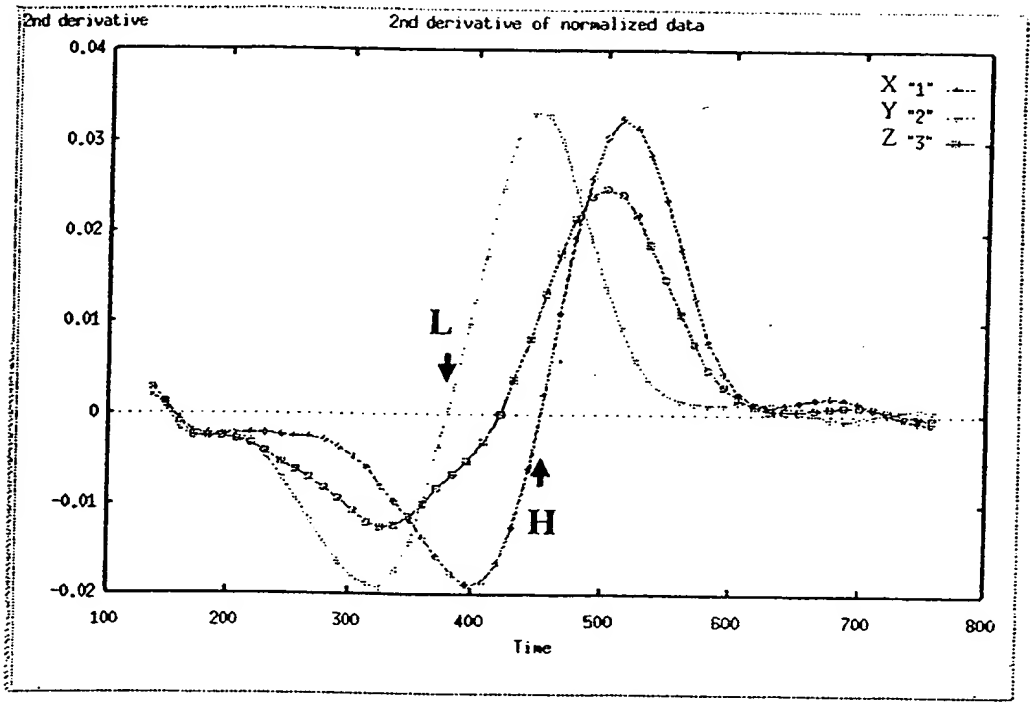
Figure 4. 2nd derivative DASH assay data for an NDUFB4 gene polymorphism

('H' and 'L' indicate points of maximum denaturation rates for matched and mismatched probe-target duplexes respectively. X, Y, and Z are the sample DNAs).

a. Results for the 'C Probe'



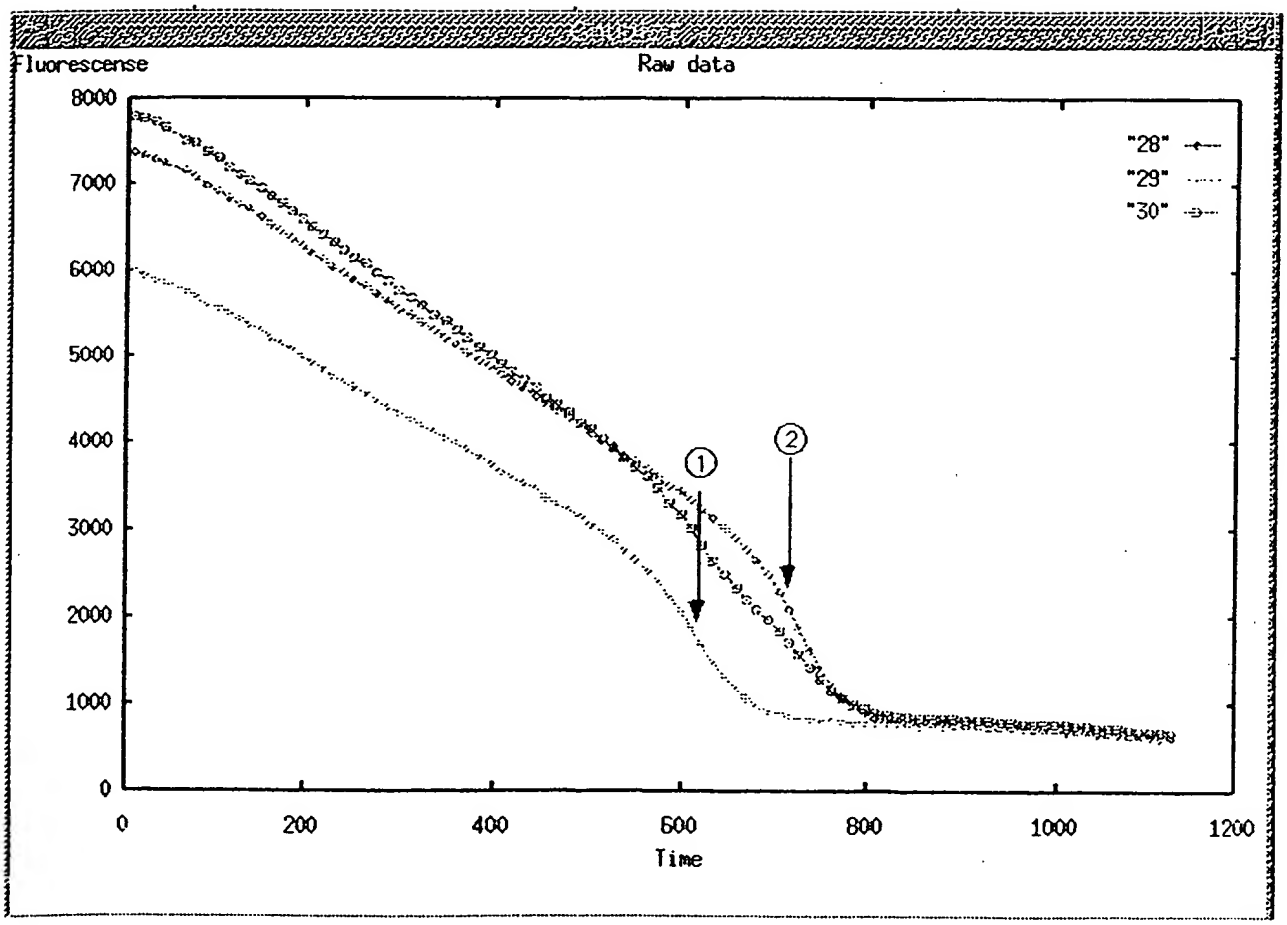
b. Results for the 'T Probe'



7. Appendix

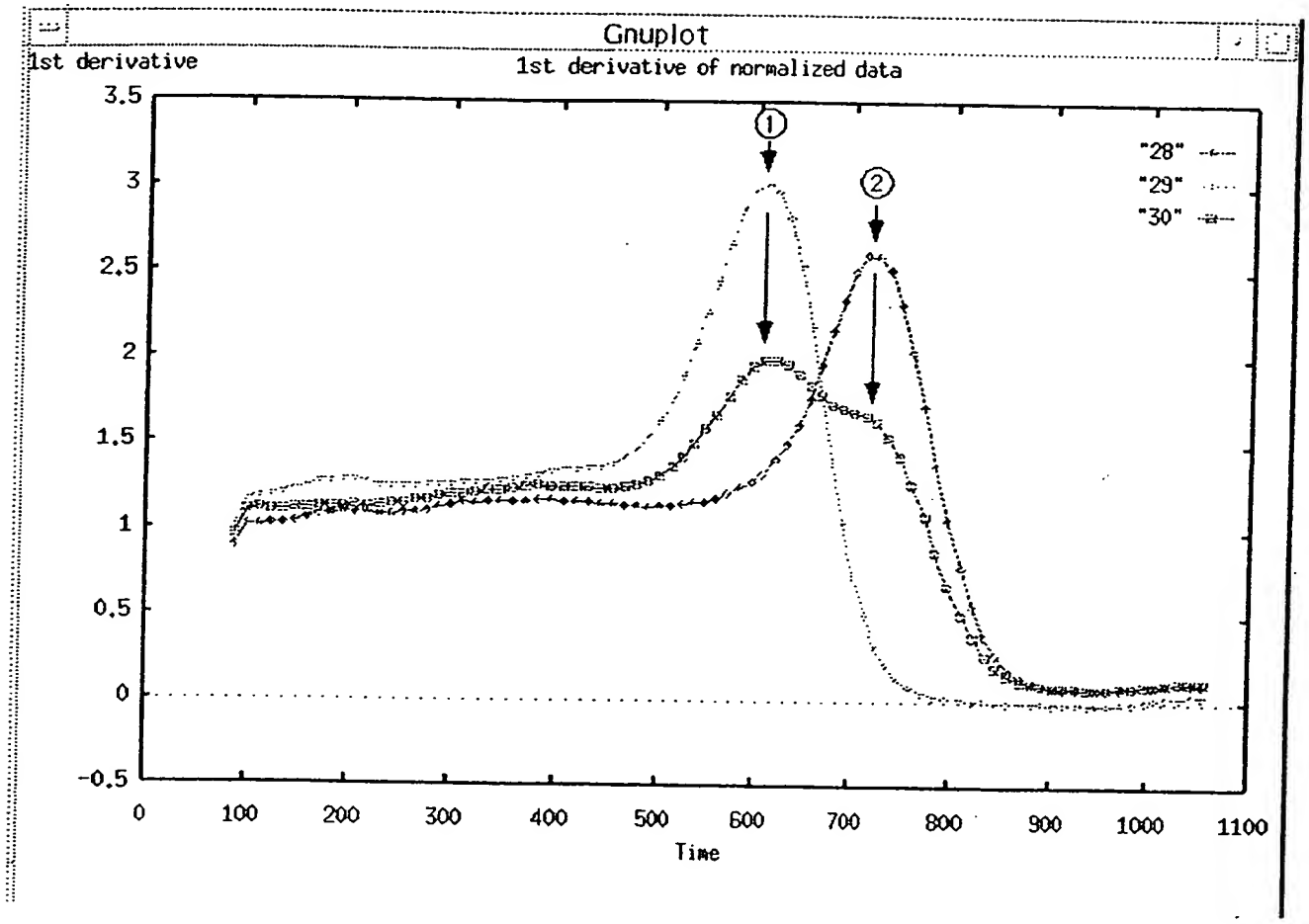
7.1. Example Graphs

7.1.1. Primary data



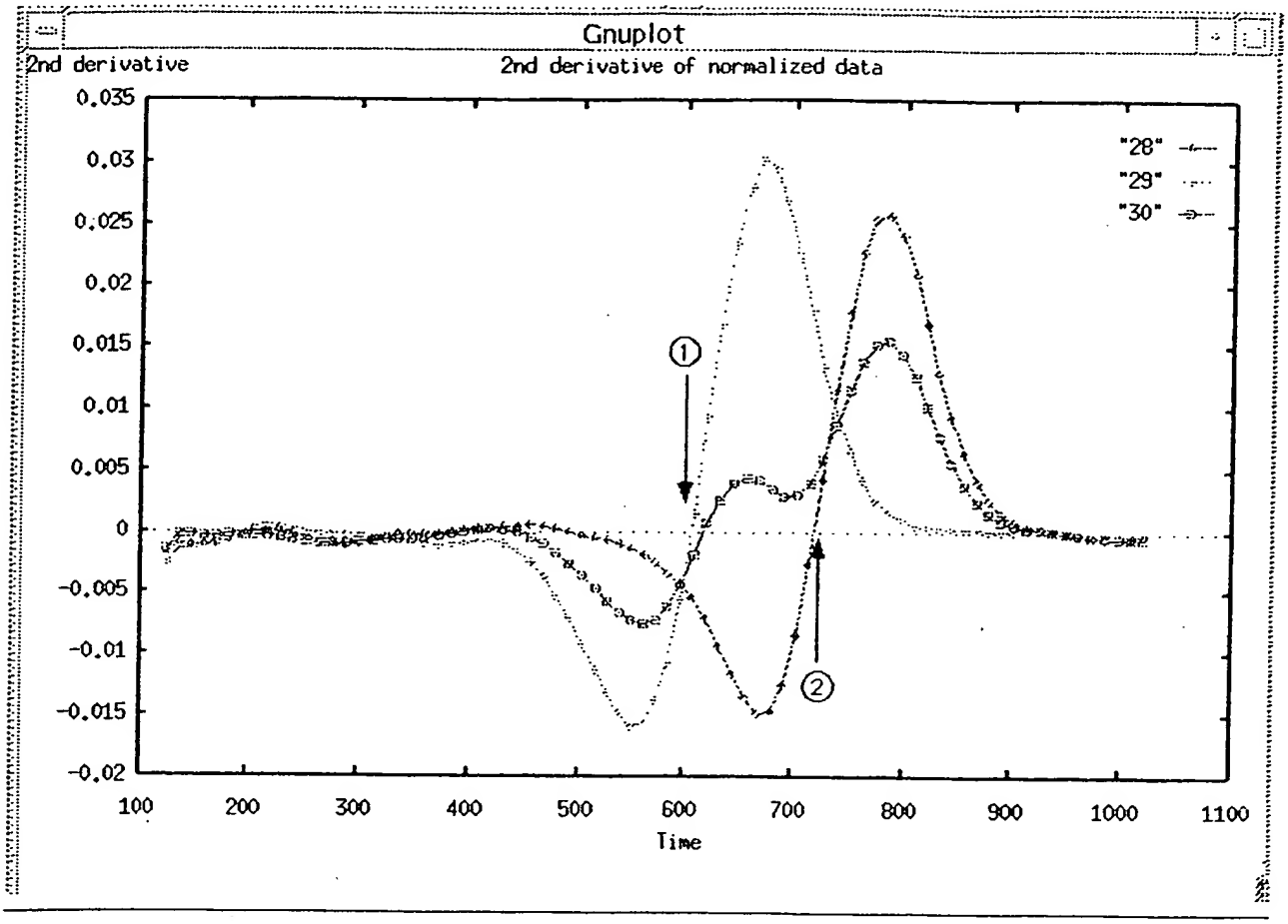
The above graph shows data for 3 different DNA samples assayed with one allele specific probe. Samples "28, 29, and 30" illustrate typical results for homozygous match, mismatch, and heterozygous samples respectively. Notice that the heterozygous sample 30 exhibits characteristics of both the match and mismatch curves. (1) Tm of mismatched probe/target duplex. (2) Tm of matched probe/target duplex.

7.1.2. Negative First derivative



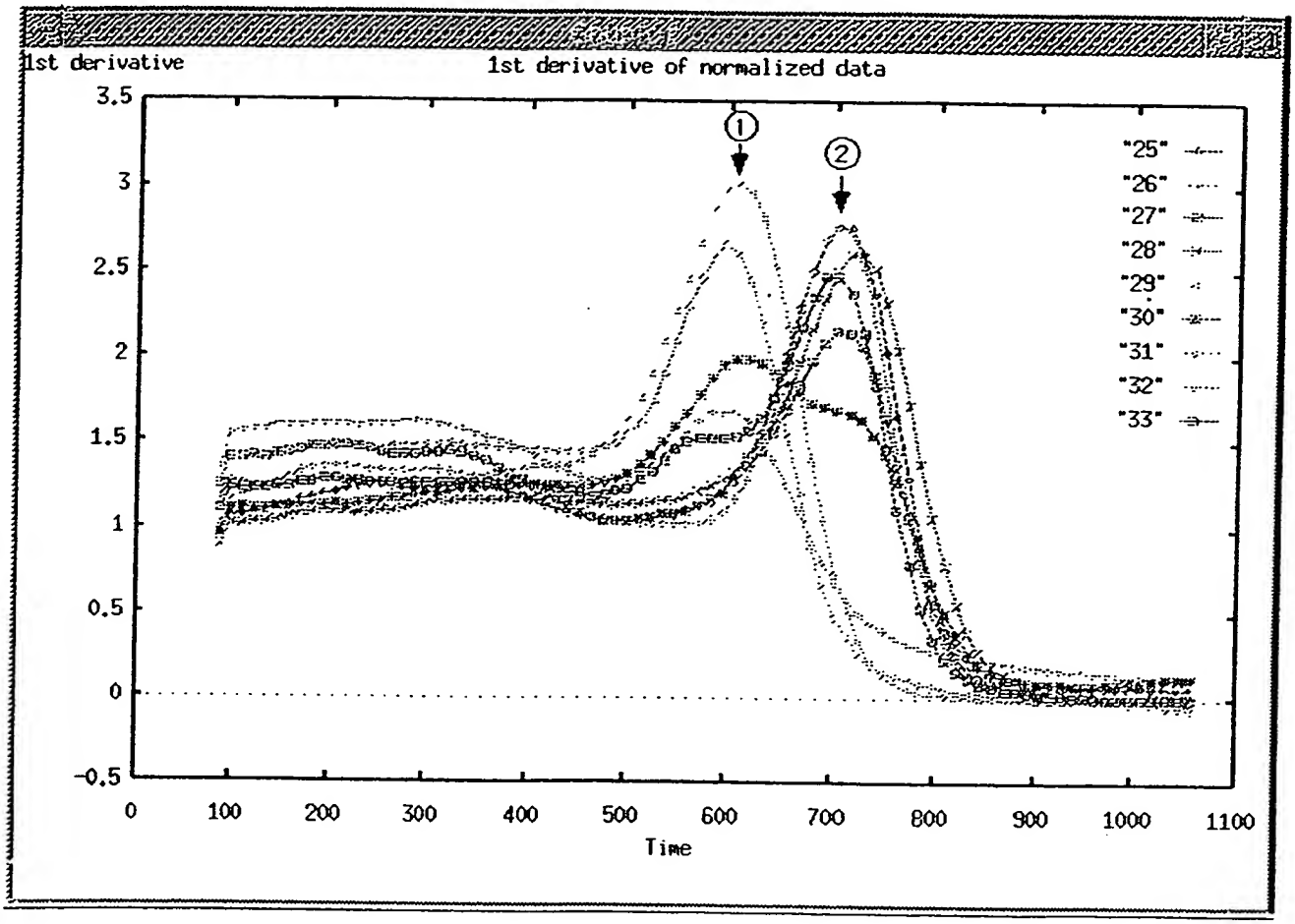
The negative first derivative of three DNA samples are shown. The DNA samples are probed with one allele specific probe. Samples 28, 29, and 30 are homozygous match, homozygous mismatch, and heterozygous, respectively, for the probe allele. (1) Tm of mismatched probe/target duplex. (2) Tm of matched probe/target duplex.

7.1.3. Second derivative



Second derivative data is shown for 3 DNAs. Samples 28, 29, and 30 are homozygous match, homozygous mismatch, and heterozygous. (1) Tm of mismatched probe/target duplex. (2) Tm of matched probe/target duplex.

7.1.4. Normalisation of Tms by Hepes for three different variations



Samples 25-27, 28-30, and 31-33 are negative first derivative sets of data for three different variations. Each set was probed with a locus specific 15mer allele specific oligonucleotide probe corresponding to the respective variation being assayed. The G+C content of these probes varied from 40% to 70%. According to melting temperature theory the Tm's of the probe/target duplexes should vary between these different sequences, but the DASH assay conditions with Hepes buffer normalize the data to fixed Tm values. (1) Tm of mismatched probe/target duplex. (2) Tm of matched probe/target duplex.

CLAIMS

1. A method of detecting DNA variation by monitoring the formation or dissociation of a complex consisting of :-

(a) a single strand of a DNA sequence containing the locus of a variation,

(b) an oligonucleotide or DNA analogue probe specific for one allele of the variation and capable of hybridising to the single strand (a) to form a duplex,

(c) a marker specific for the duplex structure of (a) plus (b) which forms a complex with the said duplex and reacts uniquely when interacting within the duplex,

which comprises continually measuring an output signal indicative of interaction of the marker with duplex formed from the strand (a) and probe (b) and recording the conditions at which a change in reaction output signal occurs which is attributable to formation or dissociation of the complex and is thereby correlated with the strength with which the probe (b) has hybridised to the single strand (a).

2. A method of detecting DNA variation which comprises bringing together

(a) a single strand of a DNA sequence containing the locus of a variation,

(b) an oligonucleotide or DNA analogue probe specific for one allele of the variation and capable of hybridising to the single strand (a) to form a duplex,

(c) a marker specific for the duplex structure of (a) plus (b) which forms a complex with the said duplex and reacts uniquely when interacting within the duplex,

the components (a), (b) and (c) being brought together under conditions in which :

EITHER (i) the component (a) hybridises to component (b) and the complex is formed with component (c)

OR (ii) the components (a) and (b) do not hybridise and the complex with component (c) is not formed,

thereafter steadily and progressively adjusting the conditions of the environment, respectively,

EITHER (i) to denature the formed duplex and cause dissociation of the complex,
OR (ii) to cause formation of the duplex and resulting complex,
and continually measuring an output signal indicative of the extent of hybridisation of (a) and (b) and resulting complex formation with (c)
and recording the conditions in which a change of output signal occurs which is indicative of, respectively (i) dissociation of the complex or (ii) formation of the complex.

3. A method of detecting DNA variation which comprises forming a complex consisting of:

(a) a single strand of a DNA sequence containing the locus of a variation,

(b) an oligonucleotide or DNA analogue probe specific for one allele of the variation hybridised to the single strand (a) to form a duplex, and

(c) a marker specific for the duplex structure of (a) plus (b) and which reacts uniquely when interacting within the duplex, and

continually measuring an output signal of the extent of the resulting reaction of the marker and the duplex whilst steadily increasing the denaturing environment containing the complex, and recording the conditions at which a change in reaction output signal occurs (herein termed the denaturing point) which is attributable to dissociation of the complex and is thereby correlated with the strength with which the probe (b) has hybridised to the single strand (a).

4. A method of detecting DNA variation which comprises bringing together

(a) a single strand of a DNA sequence containing the locus of a variation,

(b) an oligonucleotide or DNA analogue probe specific for one allele of the variation and capable of hybridising to the single strand (a) to form a duplex,

(c) a marker specific for the duplex structure of (a) plus (b) and which reacts uniquely when interacting within the duplex,

the components (a), (b) and (c) being brought together prior to formation of the defined complex and under conditions in which (a) and (b) do not hybridise, and steadily adjusting the conditions of their environment to cause formation of the duplex and resulting complex, and

measuring an output signal indicative of the occurrence of hybridisation of (a) and (b) (herein termed the annealing point).

5. A method according to any of claims 1 to 4, which comprises forming a series of two or more complexes of the kind defined, each with a probe specific for a different allele of the variation, and observing their respective denaturing or annealing conditions (e.g. denaturing or annealing temperatures) so as to distinguish alleles of the variation plus the homozygous or heterozygous state if appropriate.

6. A method as defined in any of claims 1 to 5, in which the marker is one which fluoresces when intercalated in double stranded DNA.

7. A method according to claim 6, in which the denaturing or annealing point is determined by reference to the first derivative of the fluorescence measurement curve.

8. A method according to claim 6, in which denaturing or annealing point is determined by reference to the second derivative of the fluorescence measurement curve.

9. A method according to any of the preceding claims, in which the single strand is attached to a support material.

10. A method according to claim 9, in which attachment is by a biotin/streptavidin type interaction.

11. A method according to any of the preceding claims, in which the complex is formed by adding the probe and marker to the single strand in an appropriate buffer solution.

12. A method according to claim 10, in which the buffer solution is Hepes buffer.

13. A method according to any of the preceding claims, using a fluorescent intercalating dye, in which the dye is SYBR Green I.

14. A method according to any of the preceding claims, in which the single strand is derived from a double stranded DNA product of PCR amplification of a target sequence.

15. A method according to claim 14, in which the PCR product is at least 100 base pairs in length.

16. A method according to claim 14, in which the PCR product is from 40 to 100 base pairs in length.

SEQUENCE LISTING

<110> HYBAID Ltd
BROOKES, ANTHONY J

<120> DETECTING DNA VARIATION

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/03329

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 36096 A (JENSEN MARK ANTON ;TSENG SUSAN YEN TEE (US); DU PONT (US); BASS JA) 20 August 1998 (1998-08-20) see whole doc. esp. claims and examples	1-16
X	EP 0 478 319 A (TOKYO SHIBAURA ELECTRIC CO) 1 April 1992 (1992-04-01) see whole doc. esp. claims	1-16
Y	YGUERABIDE J ET AL: "Quantitative fluorescence method for continuous measurement of DNA hybridization kinetics using a fluorescent intercalator" ANALYTICAL BIOCHEMISTRY, vol. 228, - 1 July 1995 (1995-07-01) pages 208-220, XP002107647 the whole document	1-16

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 February 2000	06/03/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Müller, F

Form PCT/ISA/210 (second sheet) (July 1992)

International Application No
PCT/GB 99/03329

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WATTS H J ET AL: "REAL-TIME DETECTION AND QUANTIFICATION OF DNA HYDRIDAZATION BY AN OPTICAL BIOSENSOR" ANALYTICAL CHEMISTRY,US,AMERICAN CHEMICAL SOCIETY. COLUMBUS, vol. 67, no. 23, 1 December 1995 (1995-12-01), pages 4283-4289, XP000540115 ISSN: 0003-2700 see whole doc. esp. discussion	1-16
A	WO 93 10266 A (US GOVERNMENT) 27 May 1993 (1993-05-27) the whole document	
A	STIMPSON D I ET AL: "REAL-TIME DETECTION OF DNA HYBRIDIZATION AND MELTING ON OLIGONUCLEOTIDE ARRAYS BY USING OPTICAL WAVE GUIDES" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA,US,NATIONAL ACADEMY OF SCIENCE. WASHINGTON, vol. 92, July 1995 (1995-07), pages 6379-6383, XP002912130 ISSN: 0027-8424	

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WO 9310266 A	27-05-1993	AU 3140293 A	15-06-1993